REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE
Breast Cancer Predoctoral Training Program

5. FUNDING NUMBERS
DAMD17-94-J-4111

6. AUTHOR(S)
Stuart A. Aaronson, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai School of Medicine
New York, New York 10029-6574

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The Breast Cancer Predoctoral Training Program (BCPTP) at the Mount Sinai School of Medicine has entered its third year. Four new students were added to the program, bringing to a total of six students who are funded by the training grant, and eight students who are currently actively involved in the BCPTP. The organization of a biweekly trainee luncheon series has stimulated discussion of issues relevant to breast cancer research among both trainees and faculty. The training program has served the dual function of encouraging the predoctoral students to focus on the issues of breast cancer in their research and training and at the same time has served as a focal point to bring various Mount Sinai faculty together to stimulate collaborations and research in the area of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Training, Multidisciplinary, Oncogenes, Growth Factors, Signal Transduction, Epidemiology, Humans, Anatomical Samples, Breast Cancer	52
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

GRANT NUMBER DAMD17-94-J-4111

TITLE: Breast Cancer Predoctoral Training Program

PRINCIPAL INVESTIGATOR: Stuart A. Aaronson, M.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, New York 10029-6574

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

SA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

SA In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

_____

PI - Signature Date

TABLE OF CONTENTS

ANNUAL REPORT - YEAR 3
BREAST CANCER PREDOCTORAL TRAINING PROGRAM
(07/01/96-06/30/97)

INTRODUCTION

The Breast Cancer Predoctoral Training Program (BCPTP) at the Mount Sinai School of Medicine has entered its third year with six students currently supported. The training program has served the dual function of encouraging the predoctoral students to focus on the issues of breast cancer in their research and training and at the same time has served as a focal point to bring various Mount Sinai faculty together to stimulate collaborations and research in the area of breast cancer. After some initial growing pains, the program is maturing into an exciting and stimulating one in which students and faculty alike have been excited and focused to the area of breast cancer research. Although the actual research that some of the students are performing may not directly impact on breast cancer, the other elements of the training program have been designed to educate the students concerning the important questions that need to be answered in breast cancer research and to stimulate the students to pursue careers that involve research into the underlying mechanisms responsible for human breast cancer.

BODY

Program Direction

Dr. Stuart Aaronson has continued as the Principal Investigator of the training grant and serves as the Director of the Mount Sinai BCPTP. During the first two years of the training grant, the faculty of the Derald H. Ruttenberg Cancer Center were dispersed at two geographically distinct sites. Several faculty were located in temporary space at the Rockefeller University and the remaining faculty including Dr. Aaronson were located in temporary space on the Mount Sinai campus. In the last year, the Cancer Center faculty have been centrally located in a newly opened East Building. Over 48,000 square feet of research space has been dedicated to the cancer effort at Mount Sinai and a rigorous recruitment effort is underway to expand the cancer faculty by as many as 8 new members. The consolidation of the Cancer Center in its new quarters has allowed for better organization and more focus to the BCPTP. Dr. James Manfredi, a new faculty member in the Cancer Center, has taken over as Training Coordinator. The steering committee composition remains essentially that same, however, and still reflects the multi-disciplinary nature of the training program. The members of the steering committee are shown in Appendix A.

Research Progress of Trainees

Five students were supported by the BCPTP in Year 2 of the program. In order to expand the number of students that are involved in the training program utilizing the limited resources available, it was decided that students would not be

supported by the training grant for more than two consecutive years. With this in mind, three of the students from Year 2 (Maxim Fonarev, Ulrich Hermanto, and Tara Santore) were rotated off the grant and were subsequently supported by their preceptor's own research funding. The two remaining students (Jessica Feinlieb and Wei Li) continued to be supported by the training grant in Year 3. The three vacant slots were filled by Albert Fliss, Yariv Houvras, and Sandro Santagata. During the course of Year 3, Wei Li chose to take a leave of absence from the Mount Sinai School of Medicine and was replaced on the training grant by Selvon St. Clair. A summary of the trainees supported to date by the BCPTP is shown in Appendix B. A total of eight students who are involved in the BCPTP continue their predoctoral study at Mount Sinai (Maxim Fonarev has since chosen not to continue his Ph.D. studies). A list of those students, their research topic, and the name of their preceptor is show in Appendix C. The research progress of the six currently supported students is examined in detail here.

Jessica Feinlieb

Jessica and her colleagues in the laboratory of Dr. Robert Krauss in the Department of Biochemistry recently isolated a novel gene designated *cdo* (CAM-related *d*own regulated by *o*ncogenes) from the rat and the human. Sequence analysis of cdo indicates that it is a 1242 amino acid protein containing 5 Ig domains, 3 FN type-III domains, a single pass transmembrane domain and a 256 amino acid cytoplasmic tail. Based on its sequence, *cdo* is a member of the immunoglobulin superfamily and a founding member of a new immunoglobulin/fibronectin type-III repeat subfamily. It is suspected that this gene acts as a negative growth regulator or as a possible tumor suppressor gene. This is supported by the circumstances under which *cdo* was isolated, and human *cdo*'s chromosomal location, 11q23-24, which is known to sustain loss of heterozygosity in breast, ovarian and lung carcinomas. *cdo* is expressed in primary breast cell cultures and is dramatically down-regulated or undetectable in multiple breast tumor cell lines. Additionally, *cdo* is highly expressed in the developing nervous system and the limb bud of the rat embryo. To further examine *cdo*'s possible role in differentiation, *cdo* was ectopically expressed in B104 cells, a neuroblastoma derived cell line, and PC-12 cells. This expression induced neurite outgrowth in these cells in the absence of additional differentiation-inducing stimuli. Furthermore, exposure of these cells to soluble, unclustered *cdo* also induced differentiation. This preliminary evidence suggests that *cdo* may play a substantial and pivotal role in neuronal differentiation. A role in differentiation is consistent with a possible role for *cdo* as a tumor suppressor gene.

Albert Fliss

The hsp90 chaperone machine plays a vital role in steroid receptor hormone binding as well as downstream transcriptional activation. However to date, no conclusive study has been performed to determine the role of the hsp90 chaperone machine in high affinity hormone binding to the estrogen receptor. Albert and his colleagues in Dr. Avrom Caplan's laboratory in the Department of Cell Biology and Anatomy have used the yeast *Saccharomyces cerevisiae* was developed as a model system to address this question. Previous studies have demonstrated that yeast is an appropriate *in vivo* model system, which enables the researcher to study the

activation of steroid hormone receptors in a convenient *in vivo* setting. In order to address the above question, hormone binding, 4-hydroxytamoxifen competition, and reporter gene transactivation assays were performed on yeast strains containing either a mutant hsp90 or Ydj1 (yeast homologue of E. coli dnaJ). The results from hormone binding studies demonstrated that both hsp90 and Ydj1 are required for high affinity hormone binding to the estrogen receptor. 4-hydroxytamoxifen competition assays suggest that hsp90 and Ydj1 are required for tamoxifen to act as a competitive inhibitor of hormone binding to the estrogen receptor. Interestingly, in the hsp90 mutant 4-hydroxytamoxifen potentiated hormone binding to the estrogen receptor, however, this did not result in a downstream potentiation of transactivation. In addition, results from lacZ reporter gene assays performed on the hsp90 mutant demonstrated a moderate decrease in downstream transactivation when compared to the isogenic wild type. Taken together these results suggest that both hsp90 and Ydj1 play vital roles in estrogen receptor hormone binding and downstream transactivation. Further studies will be performed in order to determine the mechanism of hsp90 and Ydj1 action in hormone-dependent estrogen receptor action. In addition, studies will be performed to determine the role of the other components of the hsp90 chaperone machine including hsp70, Sti1, CDC37, p23 and the cyclophilins in hormone dependent estrogen receptor activation. The results from the above and future studies should help further the understanding of the role played by the hsp90 chaperone machine in estrogen receptor activation.

Yariv Houvras

BRCA1 is a tumor suppressor gene mutated in hereditary breast and ovarian cancer. Approximately 45% of women with hereditary breast cancer and 75% of women with hereditary breast/ovarian cancer have mutations in BRCA1. In the laboratory of Dr. Jonathan Licht in the Brookdale Center for Molecular Biology, Yariv has focused his efforts in the past year towards understanding the biological function of the BRCA1 protein. He hypothesized that BRCA1 must interact with other proteins to carry out its function and began a yeast two hybrid screen to identify BRCA1 partner proteins. His search led him to a recently identified protein which functions as a phosphatase inhibitor as a potential partner protein for BRCA1. This phosphatase inhibitor fails to interact with tumor derived missense mutant forms of BRCA1, which suggests that this interaction has biological relevance. Studies are currently underway to verify this interaction by biochemical methods in human cells. Yariv is designing experiments to assess whether BRCA1 regulates the function of this partner protein and whether this interaction has effects in regulating the cell cycle.

Masako Osada

The ras-related protein, R-ras , was originally identified by low stringency hybridization method using a viral Ras cDNA as a probe. In the laboratory of Dr. Andrew Chan in the Derald H. Ruttenberg Cancer Center, Masako has shown that mutations similar to those found in the ras oncogene, when introduced into similar position in R-ras, activate its transforming potential. More recently, Masako has shown that R-ras induces cell adhesion through activation of integrin receptors in a

myeloid stem cell line 32D. To understand the signaling events mediated by R-ras leading to these two distinct biological phenotypes, a panel of R-ras mutants that could evoke differential biological function will be generated in order to dissect the downstream signal pathways responsible for transformation and cell adhesion. Cell adhesion plays an important role in cell growth and differentiation. Deregulation of integrin receptors have been correlated with an increase in the propensity of tumor cells to invade. Masako hopes that this project may lead to the better understanding of the molecular mechanisms of tumor metastasis.

Sandro Santagata

Sandro is working in the laboratory of Dr. Eugenia Spanopoulou in the Derald H. Ruttenberg Cancer Center and is currently analyzing the molecular mechanism of V(d)J site specific recombination which mediates the somatic assembly of antigen receptor gene segments. This process is initiated by the recombination activating proteins RAGI and RAG2 which recognize the recombination signal sequences (RSS) and cleave the DNA at the coding/RSS junction. Using a highly efficient *in vitro* system we found that Mg^{2+} enforced a physiological pattern of DNA binding and cleavage by RAG1/RAG2 whereas Mn^{2+} induced a relaxed/tolerant phenotype. Experiments with a RAG1 mutant that exhibits improved DNA binding affinity show that the protein recognizes the nonamer site and forms a transient initial recognition state. Recruitment of RAG2 converts this low affinity interaction into a stable RAG1/RAG2-RSS complex. A single point mutation in the homeodomain of RAG1 abolishes complex formation while mutations in either the heptamer or the nonamer elements drastically decrease formation of this stable complex. During the cleavage reaction the heptamer guides both nicking and hairpin formation while the nonamer is essential for hairpin formation. The precise role of Mg^{2+} in the initial stages of V(d)J recombination was analyzed by DNA binding and cleavage of phosphorothioated substrates. We found that Mg^{2+} directly mediates the initial DNA recognition of the RSS by RAGI/RAG2 as well as the subsequent cleavage reaction. Interestingly, Mg^{2+} coordination is critically dependent on a bridging oxygen present in the coding sequences flanking the heptamer motif. Sandro reasons that an understanding of the biochemistry of double strand break formation by RAG1/RAG2 may elucidate a role for the RAG proteins in the chromosomal translocations leading to oncogenesis.

Selvon St. Clair

Selvon has just recently begun working in the laboratory of Dr. James Manfredi in the Derald H. Ruttenberg Cancer Center. Selvon's research has focussed on the cdc25C gene product which plays a crucial role in the cell cycle and is responsible for providing the signal that triggers cells to enter mitosis. Selvon has been able to demonstrate that the promoter of the cdc25C gene contains a DNA binding element for the tumor suppressor protein p53. Selvon is currently in the process of establishing a transient transfection reporter system to delineate the physiological importance of the p53 binding site within the cdc25C promoter. To this end, Selvon has constructed luciferase reporter vectors containing various deletions of the cdc25C promoter. One of these constructs contains the p53 binding

element while the other possesses only the minimal promoter region of the cdc25C gene lacking the p53 binding site. Co-transfection of the reporter constructs along with a p53 expression plasmid will be performed. Studies such as these in conjunction with determining whether endogenous cdc25C expression is induced by p53 will allow Selvon to determine whether the DNA binding site in the cdc25c promoter has functional significance and, in turn, may suggest a role for p53 in the control of mitosis.

Selection of Trainees

The procedures for selection of trainees remains essentially the same as that used in Year 2. Applicants were asked to submit short descriptions of their research plans, a current transcript, and a letter of support from their preceptors. These were evaluated by the members of the steering committee (Appendix A). The principal members of the committee involved in these evaluations were Dr. Stuart Aaronson and Dr. Mary Rifkin. Dr. Aaronson as Director of the Derald H. Ruttenberg Cancer Center is quite familiar with Mount Sinai faculty who are engaged in cancer-related research. Dr. Rifkin plays an important role in the academic training of all graduate students at Mount Sinai and is ideally suited to judge the particular qualifications of each applicant. Criteria for selection include (1) the academic qualifications of the applicant including previous training experience (*see* Appendix E), (2) the merits of the proposed research and its potential relevance to the area of cancer and in particular breast cancer, (3) the training record, funding, and qualifications of the preceptor, and (4) the applicant's interest in pursuing a career in cancer research. As was noted earlier, three students were rotated off the training grant at the start of Year 3 and three new students were chosen to replace them. Midway through Year 3, another student left the program and she too was replaced.

Recruitment of New Trainees/Program Advertisement

Applicants for the Mount Sinai BCPTP are solicited from all students and faculty at the Mount Sinai School of Medicine. At the beginning of each academic year, an open house meeting is held to introduce prospective students to laboratories involved in cancer-related research and the BCPTP. Mailings to potential applicants as well as incoming graduate and MD/PhD students include information about the Mount Sinai BCPTP and the existence of the training grant.

Description of Training Program

BCPTP Trainee Biweekly Meetings

To focus the training program specifically on breast cancer, a biweekly luncheon meeting was established. All trainees were expected to attend. To stimulate discussion and create an informal atmosphere, lunch was served at these meetings. The purpose of these meetings was two-fold. First, Mount Sinai faculty with expertise in the area of breast cancer were invited to give didactic lectures presenting an overview of their area of expertise. Second, these meetings were used

as a forum for the students to present their own research. The schedule for these meetings is shown in Appendix G.

Since the ongoing research experience of the trainees has been primarily grounded in basic laboratory science, the invited lecture portion of these meetings was designed to give the trainees a better understanding of clinical aspects of breast cancer and to stimulate their thinking as to how their own research may help to answer some of the important questions that are currently relevant in the diagnosis and treatment of breast cancer. To that end, Dr. Mira-y-Lopez (Neoplastic Diseases) discussed the basic cell biology of the human mammary gland as a background for a presentation by Dr. Ira Bleiweis (Pathology) on the pathology of the human breast and the pathological techniques that are used in the diagnosis of breast cancer. Dr. James Holland (Neoplastic Diseases) lectured on the clinical care of breast cancer patients with a particular emphasis on his area of expertise: combination chemotherapy. Dr. Christine Eng (Human Genetics) led a lively discussion of the ethical issues involved in the genetic testing for BRCA1 and BRCA2 mutations. Finally, Drs. Mary Wolff and Jonine Bernstein (Community Medicine) discussed the environmental and genetic epidemiology, respectively, of human breast cancer. This part of the luncheon series was very well-received by the students and a number of faculty were regular participants as well. The enthusiasm of all involved has convinced us to continue this program in the coming year.

These didactic lectures were alternated with presentations of research by each of the trainees. This forum provides the opportunity for each of the students to present their current research at least twice during the academic year. These presentations were attended by members of the steering committee as well as the preceptors of all of the trainees. Students were subsequently requested to submit a short written report for evaluation by the steering committee (*see* Evaluation of Training Program).

In summary, these biweekly meetings gave a sense of cohesion and unity to the group of trainees and their preceptors, allowed for interaction and potential collaboration between the various training laboratories, and gave faculty and students alike an opportunity to become better educated about the important issues in clinical breast cancer.

Molecular Oncogenesis Research Colloquia

To foster interactions between laboratories within the Mount Sinai cancer research community and to keep both the students and faculty aware of opportunities for collaboration, a monthly Molecular Oncogenesis Research Colloquium was held. Two laboratories presented their current research each month. The schedule for the current reporting period is shown in Appendix H. The Colloquium was held in the early evening on Thursdays. Pizza and soda were served to maintain an informal atmosphere and allow for an interactive discussion. BCPTP trainees were required to attend these monthly events.

Molecular Basis of Disease Journal Club

The interdisciplinary graduate training program, Molecular Basis of Disease, sponsored a weekly journal club. Presentation at this journal club was required of all students in the program who have not yet passed their Second Level

examination which involves a demonstration of general knowledge in the form of an oral examination by a committee of faculty and a defense of a thesis proposal. Three of the students currently in the BCPTP presented at this journal club: Masako Osada, Selvon St. Clair, and Yariv Houvras. The remaining BCPTP students were required to attend and participate. The schedule for this Molecular Basis of Disease journal club is shown in Appendix I.

Recommended Seminars

There is a full and varied schedule of research seminars presented at the Mount Sinai School of Medicine. During the course of the current reporting period, particular seminars were identified and attendance was recommended for the BCPTP trainees. These seminars were chosen because the topics were relevant to the general area of cancer biology and/or breast cancer. A list of these seminars is shown in Appendix J. Once a month (twice in December), a seminar that was particularly relevant to breast cancer and the training program was identified and attendance was required for the BCPTP trainees. These required seminars are also indicated in Appendix J. On three occasions, it was arranged that the BCPTP trainees shared lunch with the seminar speaker. The speakers were Barbara Weber (12/19/96), Wen Hwa Lee (04/10/97), and Raymond White (05/14/97). This gave the students the opportunity to interact directly and as a group with the visiting scientist without the presence of faculty or other staff.

Courses

Two relevant cancer-related courses are offered by the Graduate School at Mount Sinai. The BCPTP trainees were expected to complete both of these courses. These courses were Advanced Topics in Cancer Biology, organized by the Derald H. Ruttenberg Cancer Center, and Advanced Signal Transduction, organized by the Department of Pharmacology.

Advanced Topics in Cancer Biology

In previous years, the Advanced Topics in Cancer Biology course was organized as a series of didactic lectures covering relevant topics in cancer. In the current reporting period, a new Molecular Basis of Disease overview course was organized in which ten lectures are dedicated to cancer and cancer-related topics. The Advanced Topics in Cancer Biology course was therefore re-organized to consist of journal article-based student presentations in particular topics that are relevant to cancer biology. The topics chosen for this past year were (1) Growth Factors and Receptors, (2) DNA Recombination and Repair, and (3) Apoptosis. A course outline and reading list is included in Appendix K. Except for Sandro Santagata and Albert Fliss who both had completed their course requirements by the time of their entry into the BCPTP, all of the other trainees have completed this course requirement.

Advanced Signal Transduction

The Advanced Signal Transduction course is an inter-departmental effort organized by the Department of Pharmacology. It is a lecture-based course which covers a wide range of topics, many of which are relevant to cancer (*see* Appendix L). As with the Advanced Topics in Cancer Biology course, except for Sandro

Santagata and Albert Fliss, all of the BCPTP trainees have completed this course requirement as well.

Evaluation of Training

The Graduate School at Mount Sinai requires that students meet twice a year with their advisory committees to evaluate progress both through course work and in the laboratory. The advisory committees for the BCPTP trainees are listed in Appendix D. As was noted above, trainees also present their research twice a year to members of the steering committee and trainee preceptors during the biweekly trainee luncheons. Trainees submit a written summary of their research to be evaluated by the steering committee. As part of the biweekly luncheon meetings, students are encouraged to discuss how their current research may potentially impact on the area of breast cancer and to express what interest they have in pursuing careers involving breast cancer research. The results of such evaluations have shown that the current trainees are among the best graduate students at Mount Sinai.

Faculty Collaboration

The participation of the faculty in the Mount Sinai BCPTP has led to increased discussions and collaborations, in some cases, specifically in the area of breast cancer research. Dr. Mary Wolff in the Department of Community Medicine has organized monthly luncheon meetings to discuss research initiatives involving the role of environmental and genetic influences in the development of breast cancer. These monthly meetings have led to a program project initiative led by Dr. Ze'ev Ronai in the Derald H. Ruttenberg Cancer Center. A collaboration between Dr. Mary Wolff (Community Medicine) and Dr. Paolo Fedi (Cancer Center) involving expression of erbB family members in primary human breast tumors is leading to the submission of a pilot project grant to the National Cancer Institute. Another collaboration between Dr. Wolff and Dr. James Manfredi (Cancer Center) on the role of p53 expression in the chemotherapeutic responsiveness of human breast tumors has lead to the submission of a grant proposal to a local group which funds breast cancer research. Dr. Aaronson continues to sponsor periodic meetings of a Breast Cancer Study Group in which various faculty present their research with the intent of stimulating collaborations and new grant initiatives. In summary, the BCPTP has served as a focal point to bring together Mount Sinai researchers and focus their interest on research relevant to breast cancer.

Budget

In the current reporting period, Year 3, nine trainees were supported by the BCPTP grant. Three students were supported for a full year. The remaining students were supported from two through ten months. A summary of the budget for Year 3 is shown in Appendix F.

Adherence to Statement of Work

Review progress of trainees

The academic progress of trainees is evaluated by advisory committees that are set up by the Graduate School at Mount Sinai. The progress of the trainees as it specifically relates to the BCPTP is reviewed by the steering committee through the oral presentations of the trainees at the biweekly luncheon meetings and through an annual written report. This has been successfully done for Year 3.

Select additional trainees and set up advisory committees for new trainees

Four new trainees were added to the program during Year 3 and have been successfully integrated into the BCPTP. Advisory committees are available for each of the trainees as well.

Review training program/Evaluate impact of seminars and journal club

The steering committee decided that more attention needed to be paid to educating the trainees specifically in the area of breast cancer. The biweekly luncheon meetings were initiated to perform this task. To date, these meetings have been enthusiastically received by students and faculty alike and will be continued. At least one seminar a month has been identified or organized as being relevant to the needs of the BCPTP. At the suggest of several trainees, the students themselves will assist in choosing invited seminar speakers who will address topics of direct interest to the BCPTP. The Molecular Basis of Disease journal club focussed on breast cancer in the last semester. This proved to be an important component of the BCPTP.

Organize breast cancer seminars, a journal club for trainees, with modifications if necessary

The biweekly luncheon meetings were organized to achieve this goal and have been met with enthusiasm. The format of alternating invited lectures with student presentations appears to be working well and will be continued.

Continue to recruit and advertise program

The program is entering its fourth and final year of funding. The satisfaction and interest of both faculty and students in the training program has prompted an effort to apply for funding from the National Cancer Institute in the form of a training grant to continue the BCPTP in the future.

Continue encouragement of breast cancer research among Mount Sinai faculty

The organization of monthly meetings by Dr. Mary Wolff to encourage interactions between basic science researchers and those involved in more clinical approaches will be continued. The program project initiative of Dr. Ze'ev Ronai has already stimulated discussion and collaboration among those involved. These efforts will be expanded.

CONCLUSIONS

The Mount Sinai Breast Cancer Predoctoral Training Program has had a successful Year 3. Four new students were added to the program, bringing to a total of six students who are funded by the training grant and eight students who are currently actively involved in the BCPTP. The organization of a biweekly trainee luncheon series has stimulated discussion of issues relevant to breast cancer research among both trainees and faculty. In addition, the BCPTP has served as a focal point and "launching pad" for faculty collaborations in the areas of breast cancer research. Thus, the BCPTP has stimulated significant interest in breast cancer research at the Mount Sinai School of Medicine. The program has achieved a high level of organization and a strong sense of purpose. Hence, it is anticipated that Year 4 should be successful in achieving the dual goals of enhancing the training experience the students and stimulating interest and collaborations in breast cancer research among all involved.

REFERENCES

Two manuscripts have been published in the past year by trainees involved in the BCPTP. Copies of these manuscripts are presented in Appendix M. The first, by Fang *et al..*, was submitted prior to Albert Fliss joining the BCPTP. The manuscript was used by the steering committee as evidence of Albert's productivity when considering him for entry into the program. The second manuscript by Kang *et al.* involves work done by Jessica Feinlieb while she was supported by the training grant. Accordingly, the grant is acknowledged in this manuscript.

Fang, Y., **Fliss, A. E.**, Robins, D. M., and Caplan, A. J. 1996. Hsp90 regulates androgen receptor hormone binding affinity in vivo. J. Biol. Chem. **271**:28697-28702

Kang, J. S., Gao, M., **Feinleib, J. L.**, Cotter, P. D., Guadagno, S. N., and Krauss, R. S. 1997. CDO: an oncogene-, serum-, and anchorage-regulated member of the Ig/fibronectin type III repeat family. J. Cell Biol. **138**:203-213

APPENDIX A
BCPTP Program Direction

Role	Name	Academic Rank	Department or Center Affiliation
Program Director	Stuart A. Aaronson, M.D.	Professor	Cancer Center
Training Coordinator	James J. Manfredi, Ph.D.	Assistant Professor	Cancer Center
Steering Committee	Ravi Iyengar, Ph.D.	Professor	Pharmacology
	Edward Johnson, Ph.D.	Professor	Pathology
	Robert Krauss, Ph.D.	Assistant Professor	Biochemistry
	Jonathan Licht, M.D.	Associate Professor	Molecular Biology
	Mary Rifkin, Ph.D.	Associate Professor	Molecular Biology
	Lu-Hai Wang, Ph.D.	Professor	Microbiology
	Mary Wolff, Ph.D.	Professor	Community Medicine
Graduate School Liaison	Terry Krulwich, Ph.D.	Professor/Dean	Biochemistry

APPENDIX B
Summary of Trainees Supported to Date

Trainee	Dates of Support			Total Months of Support
	Year One	Year Two	Year Three	
Jessica Feinlieb		07/01/95-06/30/96	07/01/96-06/30/97	24
Albert Fliss			10/01/96-06/30/97	9
Maxim Fonarev	07/01/94-06/30/95	07/01/95-06/30/96		24
Ulrich Hermanto	07/01/94-06/30/95	07/01/95-06/30/96	07/01/96-08/31/96	26
Yariv Houvras			07/01/96-06/30/97	12
Wei Li		07/01/95-06/30/96	07/01/96-01/31/97	19
Masako Osada			09/01/96-06/30/97	10
Selvon St. Clair			02/01/97-06/30/97	5
Sandro Santagata			07/01/96-06/30/97	12
Tara Santore	07/01/94-06/30/95	07/01/95-06/30/96	07/01/96-09/30/96	27

APPENDIX C
BCPTP Trainees, Research Project Titles, and Preceptors

Trainee	Research Project Title	Preceptor
Jessica Feinlieb (MD/PhD, Year Five)	Cdo, a novel CAM-related gene and differentiation	Robert Krauss, Ph.D. Department of Biochemistry
Albert Fliss (PhD, Year)	Hsp90 and estrogen receptor activation	Avrom Caplan, Ph.D. Department of Cell Biology and Anatomy
Ulrich Hermanto (MD/PhD, Year Six)	Receptor tyrosine kinases in breast cancer	Lu-Hai Wang, Ph.D. Department of Microbiology
Yariv Houvras (MD/PhD, Year Four)	Identification of BRCA1 partner proteins	Jonathan Licht, M.D. Brookdale Center for Molecular Biology
Masako Osada (PhD, Year Three)	R-ras and transformation	Andrew Chan, Ph.D. Derald H. Ruttenberg Cancer
Selvon St. Clair (PhD, Year Two)	p53 and mitosis	James J. Manfredi, Ph.D. Derald H. Ruttenberg Cancer
Sandro Santagata (MD/PhD, Year Four)	Mechanisms of genomic DNA rearrangements	Eugenia Spanopoulou, Ph.D. Derald H. Ruttenberg Cancer
Tara Santore (PhD, Year Six)	cAMP signalling in breast cancer	Srinivas R.V. Iyengar, Ph.D. Department of Pharmacology

APPENDIX D
BCPTP Trainee Advisory Committees

Trainee	Advisory Committee	
	Name	Department or Center Affiliation
Jessica Feinlieb	Jonathan Licht, M.D. Steven Salton, M.D.	Molecular Biology Neurobiology
Albert Fliss	Gillian Small, Ph.D. Jeanne Hirsch, Ph.D.	Cell Biology and Anatomy Cell Biology and Anatomy
Ulrich Hermanto	Robert Krauss, Ph.D. Irwin Gelman, Ph.D.	Biochemistry Microbiology
Yariv Houvras	Stuart A. Aaronson, M.D. James J. Manfredi, Ph.D. David Sassoon, Ph.D.	Cancer Center Cancer Center Molecular Biology
Masako Osada	Sandra Masur, Ph.D. Edward Johnson, Ph.D.	Cell Biology and Anatomy Pathology
Selvon St. Clair	Adolfo Garcia-Sastra, Ph.D. Stave Kohtz, Ph.D. Ze'ev Ronai, Ph.D.	Microbiology Pathology Cancer Center
Sandro Santagata	Thomas Lufkin, Ph.D. Francisco Ramirez, Ph.D. David Colman, Ph.D.	Molecular Biology Molecular Biology Molecular Biology
Tara Santore	(not available)	

APPENDIX E
BCPTP Trainee Prior Academic History

Trainee	Undergraduate Training					GRE or MCAT Scores
	Degree	Institution	Year Awarded	Major	GPA	
Jessica Feinlieb	A.B.	University Of Chicago	1992	Biology	3.00	MCAT: VB-9, PH-9, WR-O, BS-11, TOT-29
Albert Fliss	B.S.	University of Central Florida	1982	Microbiology	3.06	GRE: VRB-580, QUA-690 ANA-500
Ulrich Hermanto	B.A.	Boston University	1992	Chemistry	3.52	MCAT: VB-11, PH-10, WR-N, BS-12 , TOT-33
Yariv Houvras	B.S.	University of Michigan	1992	Biology	3.22	MCAT: VB-11, PH-10, WR-S, BS-11 , TOT-32
Masako Osada	B.S.	City College of New York	1994	Biology	3.60	GRE: VRB-350, QUA-560 ANA-510, ADV-590
Sandro Santagata	B.A.	Amherst College	1993	Neuroscience	3.55	MCAT: VB-10, PH-12, WR-R, BS-13 , TOT-35
Selvon St. Clair	B.Sc.	University of West Indies	1993	Chemistry	3.76	GRE: VRB-520, QUA-540 ANA-520
Tara Santore	B.S.	St. Francis College	1990	Biology	3.94	GRE: VRB-460, QUA-460, ANA-610, ADV-660

APPENDIX F
Budget Summary for Current Reporting Period

Trainee	Dates of Support	Stipend	Tuition and Fees	Supply Allowance	Total
Jessica Feinlieb	07/01/96-06/30/97	14,708	1,385	350	16,450
Albert Fliss	10/01/96-06/30/97	10,988	2,200	350	13,548
Ulrich Hermanto	07/01/96-08/31/96	2,102			2,109
Yariv Houvras	07/01/96-06/30/97	14,708	1,385	350	16,450
Wei Li	07/01/96-01/31/97	8,580	3,050	350	11,987
Masako Osada	09/01/96-06/30/97	12,606	3,825	350	16,790
Selvon St. Clair	02/01/97-06/30/97	6,128	2,200	350	8,680
Sandro Santagata	07/01/96-06/30/97	14,708	1,385	350	16,450
Tara Santore	07/01/96-09/30/96	3,720			3,727
	TOTALS	**88,248**	**15,430**	**2,450**	**106,191**

APPENDIX G
Schedule for BCPTP biweekly meetings

Breast cancer training grant biweekly group luncheons

The Army breast cancer training grant biweekly group luncheons will be held on Tuesdays beginning Tuesday, February 18, 1997 from 12:00-1:00 PM. The meeting will be held in Annenberg 25-51 until May 13th. The May 13th meeting and thereafter will be held in the Cancer Center's conference room, East Building 15-84. Lunch will be served.

Annenberg 25-51

February 18	Organizational meeting	
March 4	Cell Biology I: basic biology	Rafael Mira-y-Lopez (4-3194) Neoplastic Diseases
March 18	Research talks I	Yariv Houvras (4-9428) Jessica Feinlieb (4-6436)
April 1	Cell Biology II: pathology	Ira Bleiweis (4-9159) Pathology
April 15	Research talks II	Albert Fliss (4-6564) Masako Osada (5-8108)
April 29	Patient care I: diagnosis and treatment	Jim Holland (4-6361) Neoplastic Diseases

East Building 15-84

May 13	Patient care II: genetic testing and ethical issues	Christine Eng (4-3150) Human Genetics
May 27	Research talks III	Selvon St. Clair (5-8113) Sandro Santagata (5-8121)
June 10	Epidemiology II: environmental factors	Mary Wolff (4-6183) Community Medicine
June 24	Epidemiology I: genetic factors	Jonine Bernstein (4-8495) Community Medicine

APPENDIX H
MOLECULAR ONCOGENESIS COLLOQUIA

Speaker	Department or Center Affiliation	Colloquia Date	Colloquia Topic
Lu-Hai Wang, Ph.D.	Microbiology	10/10/96	Ros receptor signal transduction
Marius Sudol, Ph.D.	Biochemistry	10/10/96	WW proteins and cell signalling
Stave Kohtz, Ph.D.	Pathology	11/7/96	Regulation of myogenesis
James J. Manfredi, Ph.D.	Cancer Center	11/7/96	Tumor suppressor activity of p53
Ravi Iyengar, Ph.D.	Pharmacology	12/5/96	cAMP signalling
Paolo Fedi, M.D. Ph.D.	Cancer Center	12/5/96	ErbB-2 and erbB-3 signalling
Andrew Chan, Ph.D.	Cancer Center	01/09/97	Role of R-ras in transformation
Irwin Gelman, Ph.D.	Microbiology	01/09/97	Characterization of a novel tumor suppressor
Robert Krauss, Ph.D.	Biochemistry	02/06/97	Role of cdo in differentiation
Paul Finch, Ph.D.	Cancer Center	02/06/97	Frizzled-related antagonist of wnt action
Edward Johnson, Ph.D.	Pathology	03/06/97	Interaction of Purα and cyclin A
Jonathan Licht, M.D.	Molecular Biology	03/06/97	Transcriptional repression by PLZF
Mitch Goldfarb, Ph.D.	Molecular Biology	04/03/97	Fibroblast growth factors
Andrew Bergemann, Ph.D.	Pathology	04/03/97	Ephrins and their receptors in development
Xiangwei Wu, Ph.D.	Cancer Center	05/01/97	Novel genes involved in apoptosis
Eugenia Spanopoulou, Ph.D.	Cancer Center	05/01/97	Role of RAG1 and RAG2 in recombination
Christopher Burrow, M.D.	Nephrology	06/05/97	Kidney development
Zhen-Qiang Pan, Ph.D.	Cancer Center	06/05/97	Nucleotide excision repair and TFIIH

APPENDIX I
SCHEDULE FOR MOLECULAR BASIS OF DISEASE JOURNAL CLUB

Molecular Biology of Diseases Journal Club
Mondays, 12:15 p.m. Room 25-51
Spring Semester Topics: Breast Cancer and the Molecular Basis of Environmental Carcinogenesis.

2/10/97	Wang et al. (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin DI transgenic mice. Nature 239, 669-671. *Presenter: Andrew Bergemann*
2/24/97	Sicinski, P. et al. (1995) Cyclin DI provides a link between development and oncogenesis in retina and breast. Cell 82, 621-630. *Presenter: Yariv Houvras*
3/3/97	Halachmi, S. et al. (1994) Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science 264, 1455-1458. *Presenter: Vera Go*
3/10/97	Lydon et al. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes and Dev. 9, 2266-2278. *Presenter: Stephanie Schwartz*
3/17/97	Del Sal, G. et al. (1996) Cyclin DI and p2l/WAFI are both involved in p53 growth suppression. Oncogene 12, 177-185. *Presenter: Pallavi Sachdev*
3/24/97	Chen, J. et al. (1996) Cyclin-binding motifs are essential for the function of p2lCIP1. Molecular and Cellular Biology 16, 4673-4682. *Presenter: Hong Liu*
3/31/97	Bacus S. S. et al. (1996) Neu differentiation factor (heregulin) activates a p53-dependent pathway in cancer cells. Oncogene 12, 2535-2547. *Presenter: Caryn Chu*
4/7/97	Savitsky, K. et al. (1995) A single ataxia telangiectasia gene with a product similar to PI-3kinase. Science 268, 1749-1753. *Presenter: Masako Osada*
4/14/97	Limin, L. et al. (1997) The TSG101 tumor susceptibility gene is localized to chromosome 11 band pl5 and is mutated in human breast cancer. Cell 88, 143-154. *Presenter: Alex Chang*
4/21/97	Hakem, R. et al. (1996) The tumor suppressor gene BRCAI is required for embryonic cellular proliferation in the mouse. Cell 85, 1009-1023. Presenter: Yue Wang
4/28/97	Paulovich A.G. and Hartwell, L.H. (1995) A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell 82, 841-847. Presenter: Wilfred Holness
5/5/97	Scully et al. (1997) Association of BRCAI with Rad5l in Mitotic and meiotic cells. Cell 88, 265-275. Presenter: Joanne Hama
5/12/97	Dennisenko M. F. et al. (1996) Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. Science 274, 430-432. Presenter: Kim Lezon-Geyda
5/12/97	Bischoff et al. (1996) An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 274, 373-376. Presenter: Selvon St. Clair

APPENDIX J
BCPTP Recommended Seminars
(For seminars in bold, attendance was required for BCPTP trainees)

Speaker	Affiliation	Seminar Date	Seminar Title	Sponsoring Department or Center
Antonio Giordano, M.D. Ph.D.	Thomas Jefferson University	07/25/96	Retinoblastoma family and cyclin dependent kinases in cell cycle and cancer	Cancer Center
Chawnshang Chang, Ph.D.	University of Wisconsin-Madison	08/19/96	Androgen receptor and androgen receptor coactivator in prostate cancer	Cancer Center
Jeff Wrana, Ph.D.	Hospital for Sick Children, Toronto	10/10/96	MAD-related proteins in TGFb signalling and cancer	Cell Biology and Anatomy
Don Ingber, M.D. Ph.D.	Harvard Medical School	10/15/96	Integrin signalling, cell shape, and control of angiogenesis	Cancer Center
Savio Woo, Ph.D.	**Mount Sinai School of Medicine**	**10/22/96**	**Gene therapy for solid tumors**	**Cancer Center**
Melissa Little, Ph.D.	University of Queensland	10/24/96	The WT1 gene, development, and disease	Molecular Biology
Mark Kenny, Ph.D.	Picower Institute for Medical Research	11/08/96	Cellular responses to DNA damage	Cancer Center
Bernard Kerdelhue, Ph.D.	University René Descartes, Paris	11/11/96	Neuroendocrine disorders induced by a chemical mammary carcinogen	Cancer Center
Richard Cerione, Ph.D.	Cornell University	11/20/96	Cdc42: more questions than answers	Cancer Center
Anindya Dutta, Ph.D.	**Harvard Medical School**	**11/26/96**	**p21: an effector of the tumor suppressor p53**	**Cancer Center**
Francis Kern, Ph.D.	**Georgetown University**	**12/03/96**	**Angiogenic growth facto rs and breast cancer progression**	**Cancer Center**
Barbara Weber, M.D.	**University of Pennsylvania**	**12/19/96**	**Update on breast cancer susceptibility genes**	**Cancer Center**
Richard Youle, Ph.D.	National Institutes of Health	01/17/97	Engineering protein toxins for brain tumor therapy	Cancer Center

Klas Wiman, Ph.D.	**Karolinksa Institute**	**01/22/97**	**Restoration of the wild-type activity of mutant p53 in human tumor cells**	**Cancer Center**
Philip Hinds, Ph.D.	**Harvard Medical School**	**02/04/97**	**Cell cycle regulators in cancer**	**Microbiology**
Lawrence Feig, Ph.D.	Tufts University	02/25/97	Newly identified pathways leading into and emanating from ras proteins	Microbiology
Lili Yamasaki, Ph.D.	Massachusetts General Hospital	03/07/97	Consequences of inactivating E2F/DP transrciptional activity in vivo	Cancer Center
Jan Kitajewski, Ph. D.	**Columbia University**	**03/18/97**	**Wnt and notch genes in mammary tumorigenesis and development**	**Cancer Center**
Sally Kornbluth, Ph.D.	Duke University	03/25/97	Regulation of apoptosis	Microbiology
Konstantina Alexandropoulos, Ph.D.	Massachusetts Institute of Technology	04/01/97	Sin, a multi-adaptor assembly protein, activates and modulates c-src-dependent signal transduction	Cancer Center
Philip Tsichlis, M.D.	Fox Chase Cancer Center	04/08/97	The akt proto-oncogene: mechanisms of activation and biological function	Cancer Center
Wen Hwa Lee, Ph.D.	**University of Texas at San Antonio**	**04/10/97**	**The retinoblastoma protein**	**Molecular Biology**
Chi Dang, Ph.D.	Johns Hopkins University	04/17/97	c-myc oncogene: unique apoptotic pathways and genomic instability	Molecular Biology
Audrey Minden, Ph.D.	Columbia University	04/28/97	Signal transduction pathways mediated by the JNK family of MAP kinases	Biochemistry
David Donner, Ph.D.	Indiana University	04/29/97	Vascular endothelial cell growth factor in cancer	Cancer Center
Raymond White, Ph.D.	University of Utah	05/14/97	Insights into the molecular mechanisms of colon cancer	Dean's Series
Bruno Calabretta, M.D.	Thomas Jefferson University	05/29/97	Molecular mechanism of the bcr/abl leukomogenesis, role of the SH2and SH3 domains	Cancer Center
David Baltimore, Ph.D.	**Massachusetts Institute of Technology**	**06/11/97**	**Cell life and cell death**	**Cancer Center**
Arnold Levine, Ph.D.	**Princeton University**	**06/11/97**	**The functions of the p53 tumor suppressor gene**	**Cancer Center**

Advanced Topics in Cancer Biology
Spring, 1997

Advanced Topics in Cancer Biology will be offered in the Spring semester, 1997. Three modules will be offered. Students may take either one, two, or all three modules for credit. Each module is 1 credit. Classes meet on Tuesday and Thursday, 1:30-3:00 PM in Annenberg 18-85. This is a journal article-based class in which students take turns leading discussion of assigned journal articles. Each module will have one invited speaker who will present a research seminar that is relevant to the topic of the module. For general information, students should contact Jim Manfredi (5-8110). For details concerning the topics to be covered in each module, students should contact each of the instructors directly. An organizational meeting for the first module will be held on Tuesday, January 28 at 1:30 PM in Annenberg 18-85.

Module I: Biology of Growth Factors and Receptors
(P. Finch, 4- 8351 and P. Fedi, 4-8369)

Dates: February 4-March 6, 1997

The focus will be on the role of various growth factors and their receptors in normal biological processes as well as particular pathological states, most notably cancer. The approach to the topics will emphasize the biology of the systems being examined and thus will attempt to avoid overlap with the signal transduction course being offered concurrently.

Module II: DNA Recombination and Repair
(E. Spanopoulou, 5-8120 and Z. Pan, 5-8115)

Dates: March 11-April 10, 1997

Molecular mechanisms of DNA recombination and repair will be studied utilizing where appropriate examples of physiological or developmental relevance.

Module III: Apoptosis
(X. Wu, 4-8320 and J. Manfredi, 5-8110)

Dates: April 15-May 15, 1997

The biochemistry and molecular biology of apoptosis will be emphasized. In particular, the molecular mechanisms underlying programmed cell death will be examined, including signal transduction pathways leading to apoptosis, the bcl-2 family of proteins, and the involvement of the tumor suppressor protein p53.

Advanced Topics in Cancer Biology
February 4 - March 6, 1997

Biology of Growth Factors and Receptors
Paul W. Finch (4-8351) and Paolo Fedi (4-8369)

Articles have been selected to illustrate important aspects of growth factor signaling at the molecular, cellular, and tissue levels. Particular, but not exclusive, emphasis has been placed upon the role of growth factor signaling in tumorigenesis. Articles are meant to provide the basis for discussion. Presenters should summarize salient experimental data and ensure that they devote sufficient time to discussing the rationale for each study, and a consideration of the results in the broad context of the problem being addressed.

Tuesday, February 3

Overexpression of human insulin receptor substrate 1 induces cellular transformation with activation of mitogen activated protein kinases. T. Ito *et al.*, *Molec. Cell. Biol.*, 16: 943-951, 1996.

Neoplastic transformation induced by insulin receptor substrate 1 overespression requires an interaction with both Grb2 and Syp signaling molecules. S. Tanaka *et al.*, *J. Biol. Chem.*, 271: 14610-14616, 1996.

Thursday, February 5

Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. M. Alimandi *et al.*, *Oncogene* 10: 1813-1821, 1995.

Tuesday, February 10

Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. M. Santoro *et al.*, *Science* 267: 381-383, 1995.

Catalytic specificity of protein tyrosine kinases is critical for selective signalling. S. Zhou *et al.*, *Nature* 373: 536-539, 1995.

Thursday, February 12

Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. Y. Yang *et al.*, *J. Cell Biol.* 131: 215-226, 1995.

NDF/heregulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice. I.M. Krane and P. Leder, *Oncogene* 12: 1781-1788, 1996.

Tuesday, February 18

ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: differential expression at the endplate. N. Altoil *et al.*, *EMBO J.* 14: 4258-4266, 1995.

Induction of acetylcholine gene expression by ARIA requires activation of mitogen-activated protein kinase. J. Si *et al.*, *J. Biol. Chem.* 271: 19752-19759, 1996.

Thursday, February 20

The Epstein-Barr virus latent membrane protein 1 indices expression of the epidermal growth factor receptor. W.E. Miller *et al.*, *J. Virol.*, 69: 4390-4398, 1995.

Reversible tumorigenesis by conditional expression of the HER2/c-*erb*B2 receptor tyrosine kinase. S. Baasner *et al.*, *Oncogene*. 13: 901-911, 1996.

Tuesday, February 25

Inactivation of the type II TGF-β receptor in colon cancer cells with microsatelite instability. S. Markowitz *et al.*, *Science* 268: 1276-1277, 1995.

TGFβ1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. W. Cui *et al.*, *Cell* 86: 531-542, 1996.

Thursday, February 27

branchless encodes a drosphila FGF homolog that controls tracheal cell migration and the pattern of branching. D. Sutherland *et al.*, *Cell* 87:1091-1101, 1996.

Tuesday, March 4

The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. S. Werner *et al.*, *Science* 266: 819-822, 1994.

Modulation of keratinocyte growth factor and its receptor in reepithelializing human skin. C. Marchese *et al.*, *J. Exp. Med.* 182: 1369-1376, 1995.

Thursday, March 6

Fibroblast growth factor receptor 3 is a negative regulator of bone growth. C. Deng *et al.*, *Cell* 84: 911-921, 1996.

Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatrophic dysplasia. M.C. Naski *et al.*, *Nat. Genet.* 13: 233-237, 1996.

Advanced Topics in Cancer Biology (Spring, 1997)
Module II: DNA Recombination and Repair
Part II: DNA Repair
Lecturer: Pan (5-8115)

Topic I: Mismatch Repair Pathway in *E.coli*
Time: 03/27/97, Thursday, 1:30-3:00pm
1) Primary forms of DNA damage and their repair
2) Mechanism of methyl-directed *E.coli* mismatch repair
Article to be presented: Lahue, R.S., Su, S.-S., and Modrich, P. Requirement for d(GATC) sequences in *E.coli mutHLS* mismatch correction. *PNAS* **84:** 1482-1486. (1987).
Speaker: Lisa
Suggested readings: Modrich, P. and Lahue, R. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* **65**:101-133. (1996)

Topic II: Mismatch Repair in Eukaryotes
Time: 04/01/97, Tuesday, 1:30-3:00pm
1) Mutator/RER and microsatellite instability in Hereditary Nonpolyposis Colorectal Cancer (HNPCC)
2) Identification of eukaryotic Mut gene homologues and their role in HNPCC predisposition
3) Comparison between eukaryotic and *E.coli* mismatch repair pathways
Article to be presented: Fisher *et al.* The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **75:** 1027-1038. (1993).
Speaker: Joanne
Suggested readings: 1) Modrich, P. and Lahue, R. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* **65**:101-133. (1996); 2) Kinzler, K.W. and Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87:** 159-170. (1996)

Topic III: Nucleotide Excision Repair and Xeroderma Pigmentosum
Time: 04/03/97, Thursday, 1:30-3:00pm
1) Mechanism of *E.coli* nucleotide excision repair
2) Identification of genes responsible for xeroderma pigmentosum
3) Mechanism of nucleotide excision repair in eukaryotes
Article to be presented: Weeda et al. A presumed DNA helicase encoded by ERCC3 is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. *Cell* **62:** 777-791. (1990)
Speaker: Fabio

Suggested readings: Sancar, A. DNA excision repair. *Annu. Rev. Biochem.* **65**:43-81. (1996)

Topic IV: Transcription Coupled-Nucleotide Excision Repair and Cockayne's Syndrome
Time: 04/08/97, Tuesday, 1:30-3:00pm
1) Preferential repair of lesions in transcribed DNA strands in *E.coli* and humans
2) Mechanism of *E.coli* transcription coupled-nucleotide excision repair
3) Identification of genes responsible for Cockayne's syndrome
Article to be presented: Selby, C.P. and Sancar, A. Molecular mechanism of transcription-repair coupling. *Science* **260:** 53-58. (1993)
Speaker: Fusen
Suggested readings: Sancar, A. DNA excision repair. *Annu. Rev. Biochem.* **65**:43-81. (1996)

Topic V: DNA Damage-Responsive Cell Cycle Checkpoints
Time: 04/10/97, Thursday, 1:30-3:00pm
1) SOS response to DNA damage in *E.coli*
2) Transformation of DNA damage during the cell cycle
3) Consequences of checkpoint failure
4) MEC1 and ATM: Conservations between yeast and mammalian checkpoints
Article to be presented: Paulovich, A.G. and Hartwell, L.H. A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. *Cell* **82:** 841-847. (1995)
Speaker: Jianbo
Suggested readings: 1) Elledge, S.J. Cell cycle checkpoints: preventing an identity crisis. *Science* **274:** 1674-1672. (1996); 2) Paulovich, A.G., Toczyski, D.P. and Hartwell, L.H. When checkpoints fail. *Cell* **88:** 315-321. (1997)

Advanced Topics in Cancer Biology
Module III: Apoptosis
Part I: 4/15/97-4/29/97
X. Wu

1. Cloning of a major cystein protease Cpp32/Yama?apopin/Capase 3. its properties and inhibitors

(Nicholson et al., 1995; Tewari et al., 1995)

Show a introductory pathway and ICE family members.

2. Interaction of CED3/ICE, CED4, and CED9/Bcl-2 family proteins

Chinnaiyan, A. M.; O'Rourk, K.; Lane, B. R.; and Dixit, V. M (1997) Interaction of CED-4 with CED-3 and CED-9: A molecular frame work for cell death. Science 275: 1122-1126

Wu, D., Wallen, H. D. and Nuñez, G. (1997) Interaction and regulation of subcellular locolization of CED-4 by CED-9. Science 275: 1126-1129.

3. Cell free extracts of apoptosis: contribution of mitochondria

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jonrs, D. P., and Wang, X. (1997) Prevention of apoptosis by Bcl-2: Release of cytochrome C from mitochondria blocked. Science 275-1129-1132.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. Science 275: 1132-1136.

4. Death-domain containing proteins- TNF and FAS/APO-1 induced apoptosis.

(Chinnaiyan et al., 1995; Hsu et al., 1995; Stanger et al., 1995)

5. Death adaptors, Mach/FLICE, RAIDD

Boldin et al Cell 1996, 85: 803-815
Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-12.

Hsu, H., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495-504.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., and et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis [see comments]. Nature *376*, 37-43.

Stanger, B. Z., Leder, P., Lee, T. H., Kim, E., and Seed, B. (1995). RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell *81*, 513-23.

Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell *81*, 801-9.

Advanced Topics in Cancer Biology
Module III: Apoptosis

Apoptosis and Cancer
Jim Manfredi (East Bldg 15-23B, 5-8110)

Session #1: p53-dependent apoptosis

Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature **352:**345-347.

Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature **362:**847-849.

Howes, K. A., N. Ransom, D. S. Papermaster, J. G. Lasudry, D. M. Albert, and J. J. Windle. 1994. Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes Dev. **8:**1300-1310.

Session #2: p53: molecular mechanisms

Haupt, Y., S. Rowan, E. Shaulian, K. H. Vousden, and M. Oren. 1995. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev. **9:**2170-2183.

Ludwig, R. L., S. Bates, and K. H. Vousden. 1996. Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. Mol. Cell. Biol. **16:**4952-4960.

Session #3: E1A- and myc-dependent apoptosis and p53

Lin, H.-J. L., V. Eviner, G. C. Prendergast, and E. White. 1995. Activated H-*ras* rescues E1A-induced apoptosis and cooperates with E1A to overcome p53-dependent growth arrest. Mol. Cell. Biol. **15:**4536-4544.

Wagner, A. J., J. M. Kokontis, and N. Hay. 1994. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev. **8:**2817-2830.

Session #4: bcl-2 family: molecular mechanisms

Yang, E., J. Zha, J. Jockel, L. H. Boise, C. B. Thompson, and S. J. Korsmeyer. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell **80:**285-291.

Yin, X. M., Z. N. Oltval, and S. J. Korsmeyer. 1994. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature **369:**321-323.

Session #5: role for apoptosis in tumorigenesis and chemotherapeutic response

Knudson, C. M., K. S. Tung, W. G. Tourtellotte, G. A. Brown, and S. J. Korsmeyer. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science **270:**96-99.

Yin, C., C. M. Knudson, and T. Van Dyke. 1997. Bax suppresses tumorigenesis and stimulates apoptosis in vivo. Nature **385:**637-640.

Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957-967.

Advanced Signal Transduction

Winter-Spring 1997

This course will introduce the student to the biochemical and molecular details of cellular signaling systems. Pathways covered include G protein signaling systems, PKA and PKC; RTKs and non-receptor tyrosine kinases, small GTPases and MAPK cascades; Cytokine receptors and JAK-STAT signaling; Ser-Thr kinase receptors (TGFβ) and MAD signaling; ICE-protease family members and apoptotic signaling; and the NO-cGMP pathway. Other topics include transcription machinery and their regulation by signaling pathways and cell surface and intracellular ion channels. The course will conclude with a module on the function of signaling pathways in integrated systems. Systems to be analyzed include a sensory model: taste; a neural model: long-term potentiation of synaptic responses; and a developmental model. All lectures will use the primary literature and discuss key experiments in the field. Assigned readings will include review articles and primary literature.

Prerequiste: Students should have successfully completed Core I or prior approval of the Course Co-ordinator is required.

Time: M-W 10-12 noon
Place: BSB-244

Lecture	Date	Lecture Title	Lecturer
At the Cell Surface and Cytoplasm			
1	Jan 8	Organization of Signaling Systems	Iyengar
2	Jan 13	Growth Factors	Aaronson
3	Jan 15	Receptor Tyrosine Kinases	Wang
	Jan 20	NO CLASS- ML King Day	
4	Jan 22	Domains in RTK effectors	Sudol
5	Jan 27	Structural Aspects of SH2, SH3 and phospho-tyrosine interactions (1hr)	Weinstein
		Growth factor Signaling -I	Goldfarb
6	Jan 29	Growth factor Signaling: Receptor to Ras and Raf	Goldfarb
7	Feb 3	MAPK, JNK Pathways	Krauss
8	Feb 5	Cytokine Receptors and Interactions with JAKs	Levy
9	Feb 10	JAKS and their regulation	Sadowski
10	Feb 12	Ser-Thr Kinase Receptors (TGFβ) and MAD Signaling	Hirsch
10	Feb 18*	G protein coupled Receptors	Sealfon
11	Feb 19	Physiological Regulation of GPCRs/GRKS	Healy
12	Feb 24	GTPases and their Regulation (GAP, RGS)	Margolskee
13	Feb 26	Heterotrimeric G Proteins	Margolskee
14	Mar 3	G protein structure	Weinstein
15	Mar 5	G protein Effectors-I: cGMP-PDE, Adenylyl Cyclases	Iyengar
16	Mar 10	G protein Effectors-II: Phospholipase Cβ, PI-3 Kinase	DeVivo
17	Mar 12	G protein Effectors-III: K^+ channels	Logothetis
18	Mar 17	PKA (1hr)	Li
		PKC (1hr)	Krauss
19	Mar 19	Ser-Thr Phosphatases (1hr)	Li
20		Tyr-Phosphatases (1hr)	Salton
21	Mar 24	Intracellular Ca^{2+} stores, IP_3 Receptors,	Marks
22	Mar 26	Ca^{2+} Channels	Marks
23	Mar 31	Ligand Gated Channels (NMDA Receptors)	Landau
24	Apr 2	Apoptosis: Death Domain Receptors and TRADD	Wilk
25	Apr 7	Proteases in Signaling: ICE Family	Wilk
26	Apr 9	NO-cGMP Pathway	Healy
In the Nucleus			
27	Apr 14	Protein-DNA Interactions	Roberts
28	Apr 16	Basal Transcription Machinery	Pan
29	Apr 21	Homeobox Genes	Bieker
30	Apr 23	STATs and their Regulation	Sadowski
31	Apr 28	Fos, Jun, CREB, co-activators	Ziff
32	Apr 30	Steroid Receptors and Cross Talk	Roberts
Integrated Systems			
33	May 5	Sensory System: Taste	Margolskee
34	May 7	Cell Biol Basis of Neural Plasticity : Aplysia as a Model	Weiss
35	May 12	Synaptic Plasticity : LTP	Landau
36	May 14	Signaling in Immune Systems	Unkeless
37	May 19	Signaling in Developmental Systems	Goldfarb

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 45, Issue of November 8, pp. 28697–28702, 1996
Printed in U.S.A.

Hsp90 Regulates Androgen Receptor Hormone Binding Affinity *in Vivo**

(Received for publication, June 25, 1996, and in revised form, August 16, 1996)

Yifang Fang‡, Albert E. Fliss‡, Diane M. Robins§, and Avrom J. Caplan‡¶

From the ‡Department of Cell Biology and Anatomy, Mount Sinai Medical Center, New York New York 10029 and §Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48109

The regulation of human androgen receptor (AR) by the molecular chaperone Hsp90 was investigated using the yeast *Saccharomyces cerevisiae* as a model system. These studies were performed in strains expressing a conditional temperature-sensitive mutant allele of the *hsp82* gene, which encodes Hsp90 protein. At the restrictive temperature in the mutant, there is a decrease in hormone-dependent transactivation by the AR, although steady state levels of AR protein are unchanged. Quantitative hormone binding studies at the permissive temperature revealed the presence of both high affinity and low affinity hormone binding states. At the restrictive temperature in the *hsp82* mutant, the high affinity state was abolished, and only the low affinity state was observed. The change in hormone binding affinity was further investigated by a competition assay with the anti-androgen hydroxyflutamide. Under permissive conditions, hydroxyflutamide competes poorly for the synthetic androgen R1881, but under restrictive conditions in the *hsp82* mutant strain, hydroxyflutamide was shown to be a potent competitive inhibitor. Our findings indicate that Hsp90 participates in the activation process by maintaining apoAR in a high affinity ligand binding conformation which is important for efficient response to hormone.

Steroid hormones bind to intracellular receptors and transform them into active transcription factors (see Ref. 1 for review). Prior to ligand binding, the inactive apo-receptors are maintained in a heterocomplex with several molecular chaperones. Among these are Hsp90 and Hsp70 as well as several other proteins, some that have been characterized as peptidyl-prolyl isomerases and some that remain relatively uncharacterized. Among the latter are three proteins called p60 (2), p48 (3, 4), and p23 (5). It appears that all of these proteins are bound to steroid hormone receptors indirectly via Hsp90 (see Refs. 6–8 for reviews).

Hsp90 is required for high affinity ligand binding to several steroid hormone receptors, but with different requirements. In the case of glucocorticoid receptor (GR),[1] Hsp90 is required for high affinity ligand binding, and in the absence of the chaperone the affinity for hormone is reduced by 100-fold (9–11). High affinity hormone binding to mineralocorticoid (MR) and dioxin receptors similarly require Hsp90 (12, 13). Progesterone receptor (PR) appears to behave differently, however, since high affinity hormone binding occurs in the absence of Hsp90 at 4 °C, although not at 37 °C (4).

The AR displays similarity with PR since high affinity hormone binding (with dissociation constants (K_d) in the low nM range) was observed *in vitro* in the absence of Hsp90. Studies by Nemoto *et al.* (14) compared the affinity of hormone for AR after *in vitro* expression in rabbit reticulocyte lysates and *in vivo* expression in *Escherichia coli*. In these experiments, the *in vitro* expression results in association of AR with Hsp90, while expression in *E. coli* does not. Nemoto *et al.* (14) observed small differences in the affinity of hormone for AR in these two systems, but concluded that these were not the consequence of Hsp90 binding.

We have utilized the yeast system as an *in vivo* environment in which to study the role of Hsp90 in AR regulation. Previous studies revealed that AR function is hormone dependent in yeast cells, suggesting conservation in the regulatory machinery that controls activation (15, 16). Furthermore, yeast strains expressing reduced amounts or mutant forms of Hsp90 are defective for transactivation by estrogen, GR, MR, and PR (11, 17–19). Using yeast strains expressing a mutant form of a gene encoding Hsp90, we tested how Hsp90 loss of function affected AR activity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains used in this study are described in Table I. Yeast cultures were grown under standard conditions in medium containing 0.67% yeast nitrogen base (Difco), 2% dextrose, and appropriate amino acid supplements. Transformation of plasmids into yeast cells was performed according to method described by Gietz *et al.* (20). Plasmids that express the AR (pARU and pARH) were derived from pG1-hAR (16). pARU was constructed by inserting a blunt-ended *URA3* gene fragment into *Eco*RV-linearized pG1-hAR (within the *TRP1* gene). pARH was constructed by inserting a blunt-ended *HIS3* gene fragment into *Eco*RV-linearized pG1-hAR. pPGKareLacZC contains the *lacZ* gene under control of three cis-acting androgen response elements as described by Purvis *et al.* (15).

β-Galactosidase Activity Assay—Yeast cultures were grown to the log phase and incubated at 25 or 37 °C for 30 min prior to addition of dihydrotestosterone (DHT) for 1 h. Assays for β-galactosidase activity in whole cell extracts were as described previously (16).

Hormone Binding Assays—Yeast cells were grown to early log phase (A_{600} = 0.2) and preinucbated at 25 or 37 °C in 1-ml aliquots for 30 min. ^{3}H-R1881 (New England Nuclear) was added to the cultures (usually diluted 1:20 or 1:50 with unlabeled R1881 in ethanol) in the presence and absence of a 167-fold excess of DHT. The cells were further incubated for 90 min, washed three times with 1 ml of water, and counted in 5 ml of scintillation fluid in a scintillation counter. Specific binding was determined by subtracting the counts/min from samples incubated with DHT (giving background counts/min) from samples incubated without DHT. The background counts/min were approximately 5% of total counts.

Competition assays were performed by incubating yeast cells with

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported in part by National Institutes of Health Grant DK-49065 and by the Alexandrine and Alexander Sinsheimer Fund. To whom correspondence should be addressed: Dept. of Cell Biology and Anatomy, Mount Sinai Medical Center, One Gustave Levy Place, New York, NY 10029. Tel.: 212-241-6563; Fax: 212-860-1174; E-mail: caplan@msvax.mssm.edu.

[1] The abbreviations used are: GR, glucocorticoid receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; DHT, dihydrotestosterone; AR, androgen receptor; NTA, nitrilotriacetic acid.

TABLE I
Yeast strains used

Strain[a]	Genotype	Source
W3031b	*α ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100*	Thomas and Rothsthein (37)
ACY44	*α ade2 leu2 his3 trp1 ura3 can1* pG1-hAR pPGKareLacZI	Caplan *et al.* (16)
GPDHsp82	*α ade2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) (hsp82::LEU2) (pep4::HIS3)* pTGPDHsp82	Chang and Lindquist (21)
GPDHsp82^{FP}	*α ade2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) (hsp82::LEU2) (pep4::HIS3)* pTGPD-H-Hsp82	Chang and Lindquist (21)
ACY88	GPDHsp82 with pARU	This study
ACY89	GPDHsp82^{FP} with pARU	This study
P82a	**a** *ade2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) (hsp82::LEU2)* pTGPDHsp82	Nathan and Lindquist (19)
G170Da	**a** *ade2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) (hsp82::LEU2)* pTGPpd/T1–101	Nathan and Lindquist (19)
ACY98	P82a with pARH	This study
ACY99	G170Da with pARH	This study
ACY100	P82a with pARH & pPGKareLacZC	This study
ACY101	G170Da with pARH & pPGKareLacZC	This study

[a] All strains are derived from W303.

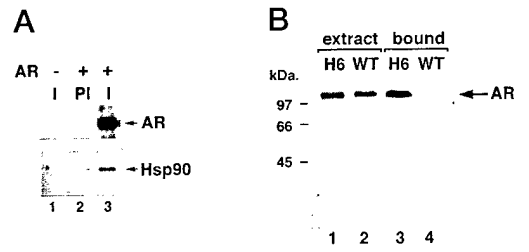

FIG. 1. **Interaction of AR with yeast Hsp90.** A, Immunoprecipitation of AR from yeast whole cell extracts with immune antisera (*lanes 1* and *3*) and preimmune antisera. Extracts were prepared from strains expressing AR (ACY44; *lanes 2* and *3*) or not expressing AR (W3031b). Western blot analysis of the immunoprecipitates was performed with antiserum against AR (*upper panel*) and Hsp90 (*lower panel*). Data are from three nonconsecutive lanes from the same gel and Western blots. *B*, affinity purification of His_6-Hsp90 from yeast strains expressing the AR and His_6-fusion of Hsp90 (*H6*, ACY89) or wild type Hsp90 (*WT*, ACY88). Samples of whole cell extracts (*lanes 1* and *2*) and eluates from NTA-agarose beads (*lanes 3* and *4*) were probed with antisera against AR.

100 nM ^{3}H-R1881 and 0–500 μM hydroxyflutamide (Scherring-Plough, stored in ethanol). The cells were washed three times in water and counted in 5 ml of scintillant. Counts/min for each sample are presented as a percentage of the counts/min from cells incubated without hydroxyflutamide. All results are the mean of three to five independent experiments.

Immunoprecipitation of AR and Affinity Isolation of Hsp90—For immunoprecipitation of AR, cells were grown to the log phase, harvested by centrifugation, and washed with buffer A (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 15 mM $MgCl_2$, 20% glycerol, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg each of leupeptin, pepstatin, aprotinin, and chymostatin). Extracts were prepared from cells in buffer A by glass bead lysis (five 30-s bursts in a BeadBeater (Biopsec products), cooling on ice for 30 s between each burst). Crude lysates were precleared at 4,500 × *g* for 5 min at 4 °C and cleared at 100,000 × *g* for 20 min at 4 °C in a TLA45 rotor. Triton X-100 was added to 0.1%, and the protein concentration was adjusted to 3 mg/ml. Immunoprecipitations were performed with 0.4 ml of extract (in buffer A plus 0.1% Triton X-100) and 3 μl of anti-AR polyclonal antibody in a 0.5-ml Eppendorf tube at 4 °C on a nutator. After 1 h, 2 μg of goat anti-rabbit IgG were added, and the reaction was continued for a further 30 min at 4 °C. The reaction tubes were centrifuged at 14,000 × *g* for 5 min at 4 °C, and supernatants were transferred to new 0.5-ml Eppendorf tubes. Protein G-Sepharose beads (20 μl of a 50% slurry in buffer A) were added, and the tubes were incubated on a nutator at 4 °C for a further 30 min. The beads were collected by centrifugation (2000 × *g* for 0.5 min), transferred to a fresh 1.5-ml centrifuge tube, and washed four times in 0.5 ml of buffer A with 0.2% Triton X-100 and once with buffer A alone. The beads were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer and boiled before gel electrophoresis of bound proteins.

Affinity isolation of His-tagged Hsp90 (His_6-Hsp90) was performed by a modified version of the procedure described by Chang and Lindquist (21). 100-ml yeast cultures were grown to log phase (A_{600} = 0.4–0.6) and washed once in lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 20 mM $NaMoO_4$, 10 mM $MgCl_2$, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg each of leupeptin, pepstatin, aprotinin, and chymostatin). The cells were pelleted, resuspended in 0.8 ml of lysis buffer, and broken in the presence of 1.5 g of 0.4-mm glass beads (w/v), and crude lysates were cleared as described above. 500 μl of protein extract (3 mg/ml) were incubated with 100 μl of Ni-NTA-agarose beads (Qiagen, 50% slurry in lysis buffer) for 15 min at 4 °C on a nutator. The beads were collected at 500 × *g* in a microcentrifuge and washed twice in lysis buffer plus 5 mM imidazole and twice in lysis buffer plus 10 mM imidazole. The beads were incubated with 1 ml of elution buffer (lysis buffer plus 150 mM imidazole) at 4 °C for 10 min. Eluted proteins were precipitated with 10% trichloroacetic acid in the presence of 8 μg/ml insulin β-chain as carrier (21).

Miscellaneous—SDS-polyacrylamide gels were prepared and run according to standard procedures. Western blots were performed as described previously (16). Antisera to AR was prepared using a mouse AR-glutathione *S*-transferase fusion protein (containing residues 133–334 of AR) in rabbits. Antisera were prepared by Lampire Biological Laboratories, Pipersville, PA. Antisera to yeast Hsp90 were described previously by Dey *et al.* (22).

RESULTS

Interaction of Hsp90 with the Androgen Receptor—The experimental basis for studying steroid hormone receptors in yeast cells relies on the conservation of the cellular machinery that controls receptor activation. This criterion is satisfied to some extent by the structural similarity in Hsp90 chaperone complexes found in yeast and higher eukaryotes (21). Furthermore, complexes between GR and yeast Hsp90 have been characterized (11, 18, 21). For the present study, we confirmed that AR could also form a specific complex with Hsp90 in wild type yeast cells.

In the experiment shown in Fig. 1*A*, the AR was immunoprecipitated from wild type yeast whole cell extracts, and the presence of Hsp90 was detected after Western blot analysis. Co-immunoprecipitation of Hsp90 with AR was considered specific since it occurred neither with preimmune sera (Fig. 1*A*, *lane 2*) nor with immune sera incubated with extracts from cells not expressing AR (Fig. 1*A*, *lane 1*). Similarly, AR protein specifically co-isolated with Hsp90 after affinity chromatography (21). For the experiment shown in Fig. 1*B*, human AR was co-expressed with an His_6-tagged Hsp90 protein (His_6-Hsp90) in yeast cells. Extracts from these cells were incubated with NTA-agarose beads to affinity purify the His_6-Hsp90 and His_6-Hsp90 binding proteins. Proteins eluted from the Ni-NTA-agarose beads were resolved by SDS-PAGE and ARs that co-isolated with His_6-Hsp90 were detected by Western blot analysis (Fig. 1*B*, *lane 3*). In a similar experiment with non-tagged wild type Hsp90, neither Hsp90 nor AR were bound to Ni-NTA-agarose beads (Fig. 1*B*, *lane 4*).

These data confirm that the interaction between Hsp90 and AR occurs in yeast, as it does in higher eukaryotes (14, 23, 24). We next tested how loss of Hsp90 function affected AR activation using yeast strains expressing a mutant *hsp82* gene.

Transactivation by the Androgen Receptor in Wild Type and

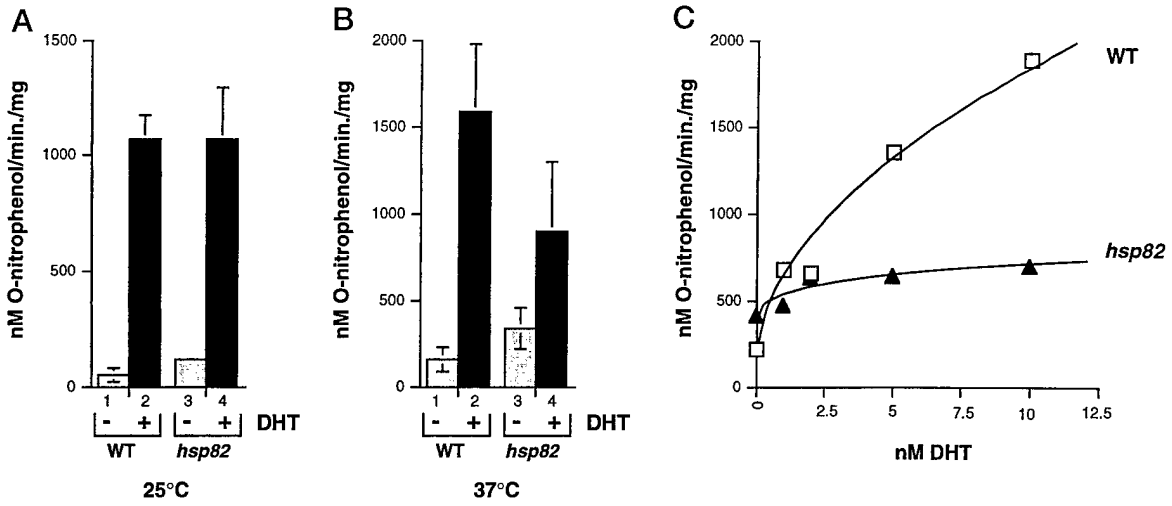

FIG. 2. **Hormone-dependent activation of AR in wild type and *hsp82* mutant yeast.** *A*, Wild type (*WT*, ACY100) and *hsp82* mutant (ACY101) yeast strains expressing the AR were incubated without (*lanes 1* and *3*) or with (*lanes 2* and *4*) 100 nM DHT for 1 h at 25 °C. β-Galactosidase activity was measured in whole cell extracts. *B*, as in *A* but at 37 °C. *C*, activation of AR at subsaturating concentrations of DHT at 37 °C for 1 h in wild type (*WT*, ACY100) and *hsp82* mutant (ACY101) yeast strains.

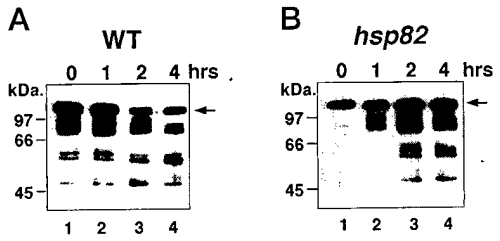

FIG. 3. **Stability of AR protein in yeast at 37 °C.** Wild type (*A*, *WT*, ACY100) and *hsp82* mutant (*B*, ACY101) yeast strains were incubated at 37 °C for the times indicated. Western blot analysis was used to visualize the full-length AR protein (*arrow*) and AR degradation products in whole cell extracts.

hsp82 Mutant Strains—Previous studies with *Saccharomyces cerevisiae* established that Hsp90 is important for hormone dependent transactivation by estrogen, GR, MR, and PR (11, 17–19). For the present study, a yeast strain expressing a mutant version of the *hsp82* gene was used to study the role of Hsp90 protein in AR regulation. This mutant, first characterized by Nathan and Lindquist (19), is a substitution of glycine 170 for aspartate in Hsp90. Both endogenous genes encoding Hsp90 proteins (*HSC82* and *HSP82*) were deleted from this strain, and the mutant *hsp82* gene was transcribed constitutively from the glyceraldehyde-3-phosphate dehydrogenase gene promoter, present on a low copy number plasmid. The mutant strain is temperature-sensitive for growth and is inviable above 33 °C. An isogenic wild type strain, viable at all temperatures tested, was used as a control for the experiments shown below. This wild type strain is also deleted for both endogenous genes encoding Hsp90 proteins and expresses the wild type *HSP82* also from a low copy number plasmid (19).

To determine the effect of Hsp90 loss of function on AR activation, both wild type and mutant (*hsp82*) strains were transformed with two plasmids: one that constitutively expressed the human AR and another that expressed the *E. coli lacZ* gene under control of the AR. *lacZ* gene expression was hormone dependent in these strains, and induction was determined by measuring soluble β-galactosidase activity in whole cell extracts.

Growing cultures of wild type and *hsp82* cells were incubated at temperatures that were permissive (25 °C) or restrictive (37 °C) for the *hsp82* strain in the presence or absence of 100 nM DHT for 1 h. At 25 °C, similar levels of β-galactosidase were observed in both strains after hormone addition (Fig. 2*A*). Despite this similarity, the induction ratio was 2.4-fold greater in the wild type (22-fold above background) compared with the mutant (9-fold above background). This occurred because uninduced β-galactosidase levels in the mutant were consistently 2-fold higher than in the wild type. This increase was AR independent since it was observed even in the absence of the AR in these strains (data not shown). At 37 °C, induction in the mutant was reduced to less than 3-fold (Fig. 2*B*), compared with a mean 10-fold induction in the wild type. This was partly due to increased background although total β-galactosidase levels were still approximately 2-fold less in the mutant than in the wild type.

The induction difference between wild type and *hsp82* mutant strains was accentuated at subsaturating DHT concentrations; at 10 nM DHT for example, *lacZ* gene induction was 9-fold in the wild type, but less than 2-fold in the mutant (Fig. 2*C*). Thus, in comparison to the wild type, the mutant strain displayed a 3–5-fold decrease in hormone dependent activation at the restrictive temperature, depending on hormone level.

These data suggested that the AR had a decreased ability to respond to hormone upon Hsp90 loss of function. This was not, however, a result of decreasing amounts of receptor, since Western blot analysis revealed similar AR protein levels in both strains (Fig. 3) The apparent decrease in full-length AR in the wild strain was observed in other experiments; similar differences in protein turnover have been reported for GR in these strains (19).

Ligand Binding to the Androgen Receptor in Wild Type and hsp82 Mutant Strains—As described above, DHT fails to properly activate AR in the *hsp82* mutant, despite there being little change in receptor protein levels. One possible explanation for these data was that Hsp90 regulated ligand binding to the receptor. This was investigated by calculating the hormone binding affinity of AR using an *in vivo* ligand binding assay. In this experiment, growing yeast cultures were treated with ^{3}H-R1881 (a synthetic androgen) over a range of hormone concentrations at 25 or 37 °C after a 30-min preincubation at these temperatures. These studies provided a basis for direct comparison of the binding characteristics in wild type and mutant strains. Binding of labeled androgen to these cells was dependent on the presence of the AR, since in its absence there was negligible specific binding.

The binding data are complex since there appeared to be two

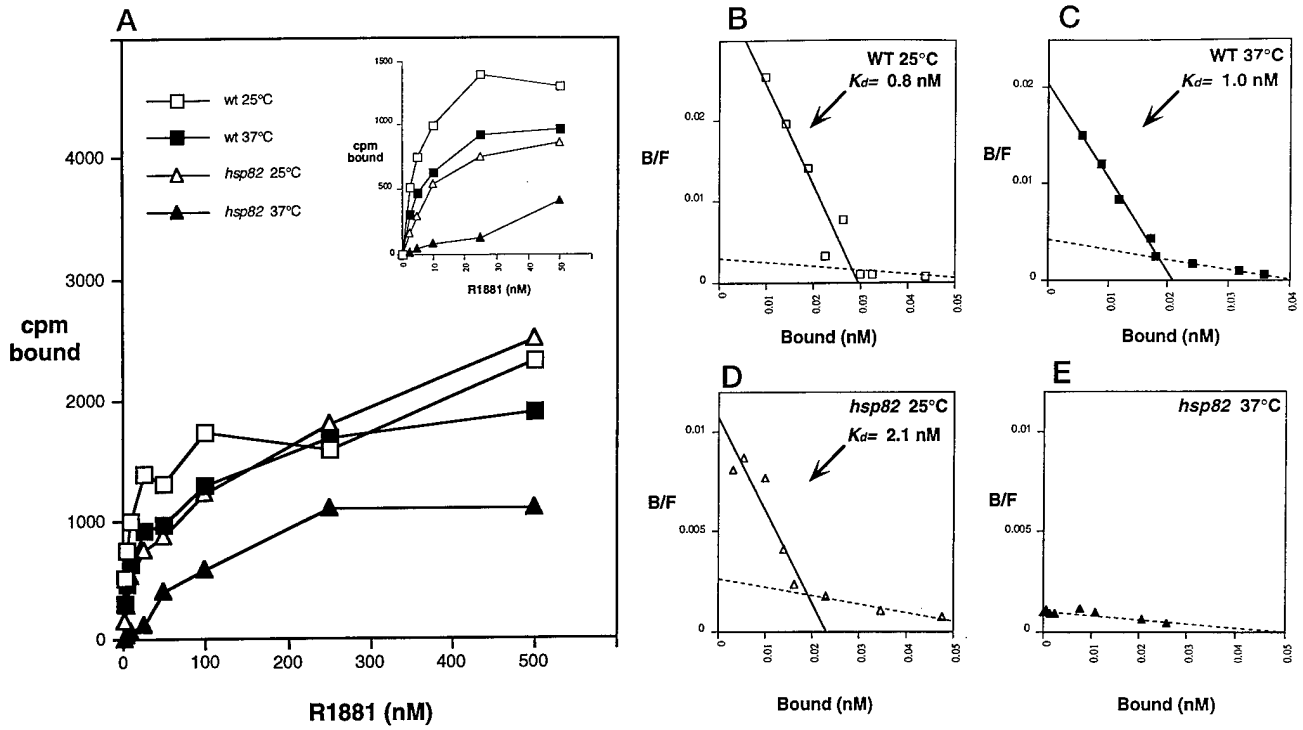

FIG. 4. **Affinity of hormone for AR in in wild type and *hsp82* mutant yeast.** *A*, titration of ^{3}H-R1881 with wild type (*wt*, ACY98) and *hsp82* mutant (ACY99) yeast cells at 25 °C and 30 °C as shown. *Inset*, enlarged view of ^{3}H-R1881 titration from 0–50 nM. *B*, Scatchard analysis of data from wild type (*WT*) strain at 25 °C. *Unbroken line*, high affinity sites; *broken line*, low affinity sites. *C*, Scatchard analysis of data from wild type (*WT*) strain at 37 °C. *D*, Scatchard analysis of data from *hsp82* strain at 25 °C. *E*, Scatchard analysis of data from *hsp82* strain at 37 °C. Symbols: □, WT at 25 °C; ■, WT at 37 °C; △, *hsp82* at 25 °C; ▲, *hsp82* at 37 °C. Each data point is the mean of three to five separate experiments.

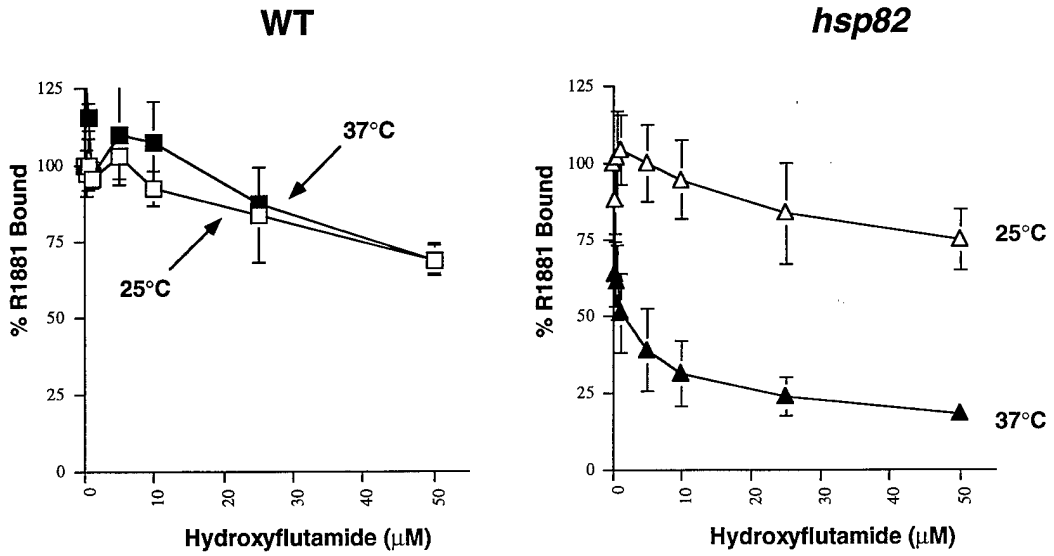

FIG. 5. **Ligand competition assay in wild type and *hsp82* mutant yeast.** Wild type (*A*, *WT*, ACY98) and *hsp82* mutant (*B*, ACY99) yeast strains were incubated with 100 nM ^{3}H-R1881 and 0–50 μM hydroxyflutamide for 1.5 h at 25 or 37 °C as indicated. Specific binding was determined in a scintillation counter and plotted as a percentage of the counts from samples incubated with 100 nM ^{3}H-R1881 in the absence of hydroxyflutamide. Each data point is the mean of three separate experiments.

binding states that differed in their affinity for the hormone. As revealed by Scatchard analysis (Fig. 4, *B–E*), the high affinity state was apparent in a linear plot between 2.5 nM and 50 nM R1881, and the low affinity state between 50 nM and 500 nM R1881 (Fig. 4, *B–E*, *dotted lines*). This was also suggested by direct inspection of the primary data, since hormone binding appeared to level off at 50 nM R1881 (except the mutant at 37 °C; see Fig. 4*A*, *inset*), but continued to rise approximately 2-fold upon a further 10-fold increase in hormone concentration. The existence of both high and low affinity states has been observed previously with retinoic acid receptors expressed in yeast cells (30).

In Fig. 4, *B–E*, the high affinity state is evident in the wild type at both temperatures (with apparent K_d values at 0.8 nM and 1.0 nM at 25 and 37 °C, respectively) but in the mutant only at 25 °C (apparent K_d = 2.1 nM). When assayed at 37 °C, the *hsp82* mutant failed to display the high affinity binding state (Fig. 4*E*) and instead, showed only the low affinity state (ap-

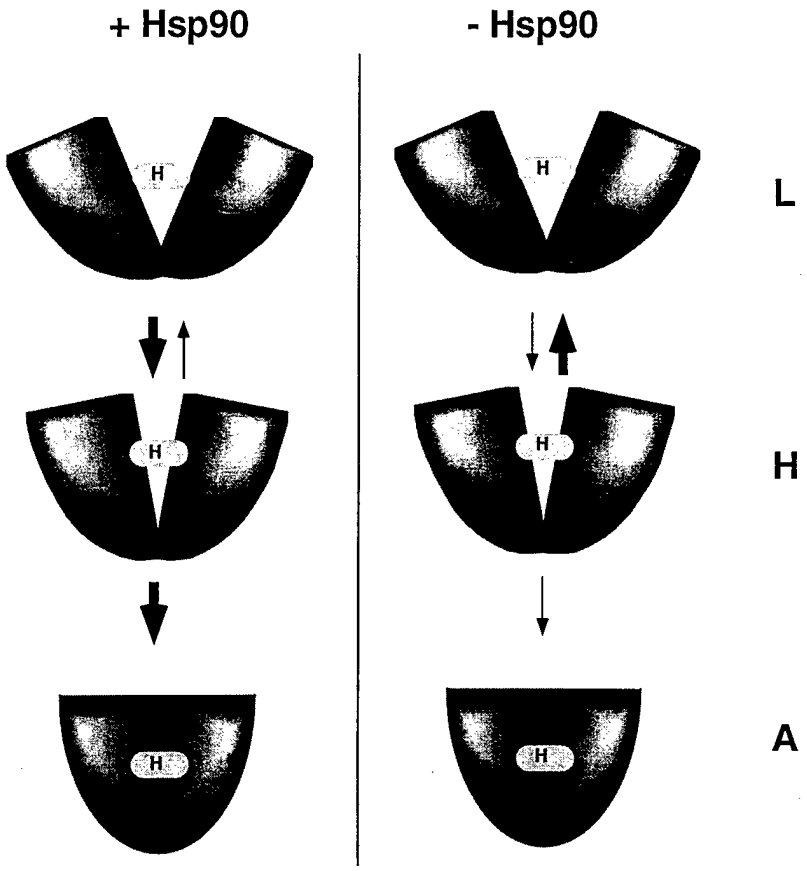

FIG. 6. **Model for the role of Hsp90 in AR hormone binding.** Model shows different conformational states of the AR ligand binding domain as a function of Hsp90 and hormone. See text for details.

parent K_d = 46.8 ± 11.2 nM for all samples). In these yeast strains, therefore, hormone binds to the AR with a similar affinity to that observed by other investigators in this (25) and in other systems (14, 26–28), but in a manner that is dependent on Hsp90 function.

The loss of high affinity R1881 binding sites in the *hsp82* mutant strain was further investigated by competition studies. These were performed using the anti-androgen hydroxyflutamide, which has a relative binding affinity for AR that is 50-fold less than R1881 (29). When tested at micromolar concentrations against the AR in yeast cells, hydroxyflutamide was an agonist in the absence of DHT and an antagonist in the presence of DHT,[2] consistent with previous studies by Wong *et al.*, (28).

In the wild type yeast strain, there was little competition for ^{3}H-R1881 over a 0–500-fold excess of cold hydroxyflutamide, either at 25 or 37 °C. In the *hsp82* mutant, however, significant competition was observed at the restrictive temperature of 37 °C, but not at the permissive temperature of 25 °C (Fig. 5). Only a 5-fold excess of hydroxyflutamide was required to reduce the binding of ^{3}H-R1881 to 50% of the starting level, whereas a 500-fold excess reduced these levels by only 25–30% in the wild type at either temperature or the mutant at 25 °C.

DISCUSSION

The results presented in this report indicate that Hsp90 maintains the AR in a high affinity hormone binding conformation. Upon Hsp90 loss of function, the AR loses the high affinity state, and as an ultimate consequence, is less active as a transcription factor in the presence of hormone (Fig. 2). Even in wild type cells, however, there exist two pools of receptor, one with high hormone affinity and one of low affinity (Fig. 4). While the nature of the low affinity state is unclear, its existence in all cells may reflect an equilibrium between receptors that are poised for activation, by having high hormone affinity, and those that are not (36). A model based upon these data is presented in Fig. 6. In this model, the apo-receptor is shown to be in equilibrium between low (*L*) and high (*H*) affinity states. A third state is shown corresponding to the active (*A*) receptor.

In the model, Hsp90 pushes the equilibrium toward the high affinity hormone binding conformation. In this state, the binding of hormone leads to further structural changes that contribute to receptor activation. Upon Hsp90 loss of function (at 37 °C in the mutant), the low affinity state is favored, and in the presence of hormone, the receptor is less able to adopt the active state. Although apparent even in wild type cells, we cannot conclude that the low affinity state measured as a consequence of Hsp90 loss of function is identical to that which occurs in the presence of functional Hsp90.

While the nature of the conformational changes in AR are unknown, there are two possible explanations that take into account a role for Hsp90 in maintaining the high affinity state. The first is that the hormone binding domain tends toward instability in the absence of Hsp90. The role of Hsp90 might then be to stabilize a conformation in AR that has a higher affinity for hormone. In this sense, Hsp90 functions as a molecular chaperone by stabilizing an otherwise unstable conformation (33).

Alternatively, the low affinity hormone binding state may reflect a kinetically trapped folding intermediate that requires not only the action of Hsp90 but also other proteins that associate with it. This could conceivably involve the peptidylprolyl isomerases known to complex with Hsp90 and steroid hormone receptors (6, 8), since these ubiquitous enzymes are well characterized in their role as catalysts of protein folding (31). In this manner, Hsp90 differs from the Hsp70 and Hsp60 (groE) chap-

[2] Y. Fang and A. J. Caplan, unpublished data.

erone machines since it integrates an enzymatic activity. The role of Hsp90 might thus be likened to a stage for the action of other chaperones, or in this case, enzymes. Indeed, it was recently demonstrated that high affinity hormone binding to PR requires the Hsp90-peptidylprolyl isomerase-p23 complex, but not the Hsp90·Hsp70·p60 complex (34).

While the results from our genetic approach strongly support a role for Hsp90 in maintaining the high affinity hormone binding state, they clearly contrast with previous studies. For example, Ohara-Nemoto *et al.* (32) and Nemoto *et al.* (14) observed only slight changes in AR hormone binding affinity in the presence or absence of Hsp90, and Xie *et al.* (26) calculated a K_d of 8 nM for R1881 binding to purified AR after refolding denatured protein (compared with K_d = 5 nM in cell extracts). An important experimental difference rests with our use of yeast as an intracellular environment in contrast to the use of purified components or cell extracts in the studies described above. A more compelling interpretation of these differences, however, is that the AR may display a conditional Hsp90 dependence for high affinity hormone binding. As previously noted, AR displays some similarity with PR since at low temperatures both receptors bind ligand with high affinity in the absence of Hsp90. At 37 °C, however, PR requires Hsp90 to maintain the high affinity steroid binding conformation (4). If such temperature dependence also applied to AR, then no significant change in hormone binding affinity may be expected at 4 °C, but would be apparent at 37 °C as was shown in Fig. 4. The hormone binding studies of Ohara-Nemoto *et al.* (32), Nemoto *et al.* (14), and Xie *et al.* (26) were all performed at low temperature.

Interestingly, such temperature dependence does not appear to be a general phenomenon among steroid hormone receptors. When assayed at low temperatures in the absence of Hsp90, there is a 100-fold decrease in the hormone binding affinity of GR, and a 1000-fold change for MR compared with studies performed in the presence of Hsp90 (10, 13). Such differences suggest that Hsp90 has specific receptor-dependent functions. In this context, Bohen and Yamamoto (18) have characterized specific mutations in Hsp90 that affect receptors differentially. For example, substitution of glutamate 431 by lysine, has little effect on the function of estrogen, MR, or PR, but profoundly affects GR. Different receptors, therefore, have specialized requirements, although whether these reflect differences in degree or in other specific needs is complex and compounded by the varied cast of Hsp90 binding proteins.

Hsp90 binds to several proteins known to be involved in signal transduction, most of which are transcription factors or protein kinases (35). What each of these has in common is that the cell must maintain them in an "off" state prior to activation. Whether the Hsp90 chaperone machine has a general role in maintaining repression of these factors or in facilitating ligand-dependent activation may yet prove to be a fundamental principle of signal transduction.

Acknowledgments—We thank Dr. S. Lindquist for yeast strains, Dr. E. Wilson for plasmids, and Dr. Sandy Ross for helpful discussions.

REFERENCES

1. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
2. Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsu, K., Madden, B., McCormick, D. J., and Toft, D. O. (1993) *Mol. Cell. Biol.* **13,** 869–876
3. Hohfeld, J., Minami, Y., and Hartl, F.-U. (1995) *Cell* **83,** 589–598
4. Smith, D. F. (1993) *Mol. Endocrinol.* **7,** 1418–1429
5. Johnson, J. L., Beito, T. G., Krco, C. J., and Toft, D. O. (1994) *Mol. Cell. Biol.* **14,** 1956–1963
6. Pratt, W. B. (1993) *J. Biol. Chem.* **268,** 21455–21458
7. Smith, D. F., and Toft, D. O. (1993) *Mol. Endocrinol.* **7,** 4–11
8. Bohen, S. P., and Yamamoto, K. R. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R., Tissieres, A., and Georgopoulos, C., eds) pp. 313–334, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Bresnick, E. H., Dalman, F. C., Sanchez, E. R., and Pratt, W. B. (1989) *J. Biol. Chem.* **264,** 4992–4997
10. Nemoto, T., Ohara-Nemoto, Y., Denis, M., and Gustafsson, J.-A. (1990) *Biochemistry* **29,** 1880–1886
11. Bohen, S. P. (1995) *J. Biol. Chem.* **270,** 29433–29438
12. Pongratz, I., Mason, G. G. F., and Poellinger, L. (1992) *J. Biol. Chem.* **267,** 13728–13734
13. Nemoto, T., Ohara-Nemoto, Y., Sato, N., and Ota, M. (1993) *J. Biochem. (Tokyo)* **113,** 769–775
14. Nemoto, T., Ohara-Nemoto, Y., and Ota, M. (1992) *J. Steroid Biochem. Mol. Biol.* **42,** 803–812
15. Purvis, I. J., Chotai, D., Dykes, C. W., Lubahn, D. B., French, F. S., Wilson, E. M., and Hobden, A. N. (1991) *Gene (Amst.)* **106,** 35–42
16. Caplan, A. J., Langley, E., Wilson, E. M., and Vidal, J. (1995) *J. Biol. Chem.* **270,** 5251–5257
17. Picard, D., Khursheed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S., and Yamamoto, K. R. (1990) *Nature* **348,** 166–168
18. Bohen, S. P., and Yamamoto, K. R. (1993) *Proc. Nat. Acad. Sci. U. S. A* **90,** 11424–11428
19. Nathan, D. F., and Lindquist, S. (1995) *Mol. Cell. Biol.* **15,** 3917–3925
20. Gietz, R. D., Schiestl, R. H., Willmes, A. R., and Woods, R. A. (1995) *Yeast* **11,** 355–360
21. Chang, H.-C. J., and Lindquist, S. (1994) *J. Biol. Chem.* **269,** 24983–24988
22. Dey, B., Caplan, A. J., and Boschelli, F. (1996). *Mol. Biol. Cell* **7,** 91–100
23. Mariovet, S., Van dijck, P., Verhoeven, G., and Heyns, W. (1992) *Mol. Cell. Endocrinol.* **88,** 165–174
24. Veldscholte, J., Berrevoets, C. A., Zegers, N. D., van der Kwast, T. H., Grootegoed, J. A., and Mulder, E. (1992) *Biochemistry* **31,** 7422–7430
25. Mak, P., Young, C. Y., and Tindall, D. J. (1994) *Recent Prog. Horm. Res.* **49,** 347–352
26. Xie, Y.-B., Sui, Y.-P., Shan, L.-X., Palvimo, J. J., Phillips, D. M., and Janne, O. A. (1992) *J. Biol. Chem.* **267,** 4939–4948
27. Chang, C., Wang, C., DeLuca, H. F., Ross, T. K., and Shih, C. C-Y. (1992) *Proc. Nat. Acad. Sci. U. S. A.* **89,** 5946–5950
28. Wong, C., Kelce, W. R., Sar, M., and Wilson, E. M. (1995) *J. Biol. Chem.* **270,** 19998–20003
29. Wakeling, A. E., Furr, B. J., Glen, A. T., and Hughes, L. R. (1981) *J. Steroid Biochem.* **15,** 355–359
30. Holley, S. J., and Yamamoto, K. R. (1995. *Mol. Biol. Cell.* **6,** 1833–1842
31. Schmid, F. X. (1993. *Annu. Rev. Biophys. Biomol. Struct.* **22,** 123–143
32. Ohara-Nemoto, Y., Nemoto, T., and Ota, M. (1991) *J. Biochem. (Tokyo)* **109,** 113–119
33. Hendrick, J. P., and Hartl, F-U. (1993) *Annu. Rev. Biochem.* **62,** 349–384
34. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. (1995) *Mol. Cell. Biol.* **15,** 6804–6812
35. Rutherford, S. L., and Zucker, C. S. (1994) *Cell* **79,** 1129–1132
36. Bohen, S. P., Kralli, A., and Yamamoto, K. R. (1995) *Science* **268,** 1303–1304
37. Thomas, B., and Rothstein, R. (1989) *Cell* **56,** 619–630

CDO: An Oncogene-, Serum-, and Anchorage-regulated Member of the Ig/Fibronectin Type III Repeat Family

Jong-Sun Kang,* Min Gao,* Jessica L. Feinleib,* Philip D. Cotter,‡ Sarah N. Guadagno,§ and Robert S. Krauss*

*Department of Biochemistry, ‡Department of Human Genetics, Mount Sinai School of Medicine, New York 10029; and §Zymed Laboratories, South San Francisco, California 94080

Abstract. Cell adhesion molecules of the Ig superfamily are implicated in a wide variety of biological processes, including cell migration, axon guidance and fasciculation, and growth control and tumorigenesis. Expression of these proteins can be highly dynamic and cell type specific, but little is known of the signals that regulate such specificity. Reported here is the molecular cloning and characterization of rat CDO, a novel cell surface glycoprotein of the Ig superfamily that contains five Ig-like repeats, followed by three fibronectin type III–like repeats in its extracellular region, and a 256-amino acid intracellular region that does not resemble other known proteins. In rat embryo fibroblasts, *cdo* mRNA expression is maximal in confluent, quiescent cells. It is rapidly and transiently down-regulated by serum stimulation of such cells, and is constitutively down-regulated in oncogene-transformed derivatives of these cells. CDO protein levels are also dramatically regulated by cell–substratum adhesion, via a mechanism that is independent of *cdo* mRNA expression. The amount of CDO produced at the surface of a cell may therefore be governed by a complex balance of signals, including mitogenic stimuli that regulate *cdo* mRNA levels, and substratum-derived signals that regulate CDO protein production. *cdo* mRNA is expressed at low levels in most adult rat tissues. A closely related human gene maps to chromosome 11q23–24, a region that displays frequent loss of heterozygosity in human lung, breast, and ovarian tumors. Taken together, these data suggest that loss of CDO function could play a role in oncogenesis.

CELL adhesion molecules (CAMs)[1] of the Ig superfamily (IgSF) are implicated in cell–cell interactions that mediate, in part, a wide variety of developmental, physiological, and pathological processes (for reviews see Edelman and Crossin, 1991; Hynes and Lander, 1992; Brummendorf and Rathjen, 1995; Cunningham, 1995). In general, CAMs of the IgSF mediate Ca^{2+}-independent, homo- and heterophilic cell–cell adhesion. These molecules typically possess an extracellular region that consists of several Ig-like domains followed, in many cases, by a number of fibronectin type III (FNIII)–like domains. Members of the IgSF that contain both Ig-like and FNIII-like repeats (e.g., neural cell adhesion molecule (N-CAM), L1, F11/contactin, etc.) have been mainly implicated in developmental processes in the nervous system, including cell migration, neurite extension, and axon guidance and fasciculation (Hynes and Lander, 1992; Tessier-Lavigne and Goodman, 1996).

Cell–cell recognition also plays an important role in the proliferative capacity of cells both inside and outside the nervous system, and Ig/FNIII family members may be involved in this phenomenon as well. For example, cell surface N-CAM mediates contact inhibition of growth of a mouse fibroblast cell line (Aoki et al., 1991), and addition of soluble N-CAM to primary cultures of rat astrocytes specifically inhibits their proliferation (Sporns et al., 1995).

Consistent with a role for CAMs in controlling cell proliferation, a large body of evidence suggests that alterations in adhesion-mediated cell regulation may play a role in induction and/or maintenance of the neoplastic phenotype (for review see Hedrick et al., 1993). It has been known since the 1940s that cancer cells display altered adhesiveness when compared to their normal counterparts (Coman, 1944). Moreover, malignant tumors exhibit a loss of appropriate tissue architecture, altered cell shape, cell crowding, and invasiveness. Tumor cell lines, or cells transformed in vitro, show a loss of contact inhibition of growth and proliferate in an anchorage-independent

Please address all correspondence to Robert S. Krauss, Department of Biochemistry, Box 1020, Mount Sinai School of Medicine, New York 10029. Tel.: (212) 241-2177. Fax: (212) 996-7214. e-mail: rkrauss@smtplink.mssm.edu

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; C-CAM, cell–cell adhesion molecule; FNIII, fibronectin type III; N-CAM, neural cell adhesion molecule; IgSF, Ig superfamily; LOH, loss of heterozygosity; FNIII, fibronectin type III.

fashion. Although the molecular bases of these phenotypes are still poorly understood, they may arise, in part, because of loss of expression or mutational inactivation of genes that encode CAMs (Hedrick et al., 1993; Berx et al., 1996).

A candidate tumor suppressor gene, *DCC*, encodes a member of the Ig/FNIII family containing four Ig-like repeats, and six FNIII-like repeats in its extracellular region (Fearon et al., 1990; Hedrick et al., 1994; for reviews see Fearon and Pierceall, 1995; Fearon, 1996). *DCC* was isolated by positional cloning in a region of chromosome 18q that commonly displays loss of heterozygosity (LOH) in colorectal cancers. *DCC* was included in the region of allelic loss in >90% of colorectal cancers that exhibited LOH of 18q. Furthermore, loss of DCC protein expression in colorectal tumors is a negative prognostic marker of survival rate in patients with stage II and stage III disease (Shibata et al., 1996). Additional studies of *DCC* expression and allelic loss indicated that this gene may also be involved in many additional types of cancer and may play a role in cell differentiation (Fearon, 1996). DCC was also recently shown to function as an evolutionarily conserved receptor or component of a receptor for netrin-1, a secreted and membrane-associated protein that acts as a guidance cue for migrating axons in the developing nervous system (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). These data raise the possibility that loss of DCC function could alter the motility of cancer cells.

An additional IgSF member implicated in carcinogenesis is cell–cell adhesion molecule (C-CAM)/biliary glycoprotein, which contains four Ig-like domains in its extracellular region. Expression of the *C-CAM/Bgp-1* gene was down-regulated in colorectal and prostate malignancies (Kleinerman et al., 1995; Neumaier et al., 1993), and ectopic restoration of its expression suppressed tumorigenicity of cell lines derived from such tumors (Hsieh et al., 1995; Kunath et al., 1995).

It is clear from the studies cited above that CAMs of the IgSF play a role in coordinating normal cellular architecture, motility, and growth control, and that loss of function of such molecules may play an important role in multistage carcinogenesis. We have previously developed and characterized a panel of somatic cell mutant lines derived from Rat 6 embryo fibroblasts that are specifically resistant to the induction of anchorage-independent growth by various oncogenes (Krauss et al., 1992; Feinleib and Krauss, 1996; Kang and Krauss, 1996). Because the oncogene-resistant phenotype of these cells was dominant in somatic cell hybridizations, we sought to isolate genes whose expression is up-regulated in the mutant cell lines relative to a transformation-sensitive control cell line. Reported here is the molecular cloning and characterization of a rat gene designated *cdo* (CAM-related/down-regulated by oncogenes), which encodes a novel transmembrane member of the Ig/FNIII family. In rat fibroblasts, *cdo* expression is maximal in confluent, quiescent cells. *cdo* mRNA and protein levels are strongly, but transiently, reduced when such cells are stimulated to reenter the cell cycle and are constitutively down-regulated in oncogene-transformed derivatives of these cells. Furthermore, CDO protein levels are strikingly regulated by cell–substratum adhesion, with a mechanism that is independent of *cdo* mRNA expression. A closely related human gene maps to chromosome 11q23–24, a region that displays LOH in human lung, breast, and ovarian tumors. Taken together, these data suggest that loss of CDO function might play a role in oncogenesis.

Materials and Methods

Cell Culture

All Rat 6–derived cell lines were routinely maintained in DME plus 10% bovine calf serum, as previously described (Krauss et al., 1992). NIH 3T3 and NIH 3T3/*ras* cells were cultured in DME plus 5% FBS. Soft agar assays and preparative methylcellulose cultures were performed as previously described (Kang and Krauss, 1996). Cells were harvested from preparative methylcellulose cultures 48 h after inoculation into the semisolid medium, and were >98% viable as assessed by trypan blue dye exclusion and replating assays (Kang and Krauss, 1996). For serum stimulation experiments, confluent cultures of Rat 6 cells were washed twice with PBS, and fed with DME plus 0.1% calf serum. After 48 h, the cultures were refed with DME plus 20% calf serum and harvested for analysis at various time points thereafter.

RNA Isolation, Library Construction, and Screening

RNA used for cDNA library construction was prepared by the guanidine thiocyanate/cesium chloride centrifugation method (Chirgwin et al., 1979), and the poly(A)$^+$ fraction was isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). RNA used for Northern blot analyses was isolated either as described above or with the TRIzol reagent (GIBCO BRL, Gaithersburg, MD), which was used according to the manufacturer's instructions.

An oligo(dT)-primed cDNA library was constructed in λZAPII (Stratagene, La Jolla, CA) with mRNA isolated from the transformation-resistant, mutant cell line, R-1a (Krauss et al., 1992). The library was constructed with the SuperScript Choice system kit from GIBCO BRL, and was screened by differential hybridization (Johnson et al., 1987) with ^{32}P-labeled cDNA prepared from R-1a and control, transformation-sensitive PKC3-F4 cells. One clone that exhibited a greater signal with R-1a–derived cDNA, relative to PKC3-F4–derived cDNA, represented a partial cDNA for *cdo*. Clones representing a nearly full-length cDNA were isolated from a random-primed λZAPII library constructed with mRNA from Rat 6 cells. A human cDNA that is closely related to rat *cdo* was isolated by screening human fetal lung and fetal brain cDNA libraries (obtained from A. Chan and S. Aaronson, Mount Sinai School of Medicine; and Clontech, Palo Alto, CA, respectively) with the full length rat *cdo* cDNA.

DNA Sequencing and Computer Analysis

DNA sequencing was carried out by a combination of manual and automated methods. Manual sequencing was performed with a Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). Automated sequencing was performed at the Mount Sinai Molecular Biology Core Facility on an Applied Biosystems 373 computerized DNA sequencer (Foster City, CA) or at the Biotechnology Center at Utah State University (Logan, UT). Searches of the DNA databases were performed with the BLAST server service, and sequence analysis and alignments were carried out with MacVector (International Biotechnologies, Inc., New Haven, CT) and GCG software (Genetics Computer Group, Madison, WI).

RNA Analyses

Northern blot analyses were performed by fractionating total cellular or poly(A)$^+$ RNA through agarose/formaldehyde gels, blotting to nylon membranes, and hybridizing with DNA probes as described by Krauss et al. (1992).

For the study of *cdo* expression in adult rat tissues, PCR-based "exon-connection" assays were carried out (Fearon et al., 1990). A portion of the *cdo* gene was isolated from a rat genomic library (a gift of S. Salton, Mount Sinai School of Medicine) and a partial exon/intron structure deduced over a ~15-kb region. Random primers and reverse transcriptase were used to generate cDNA from total RNA derived from rat tissues. PCR primers (5′-CACACAGTCAGAAGCGTTCCTC-3′ [sense], and 5′-

AGGATAAGAGGCTACCACTGGG-3′ [antisense]) directed against sequences from separate exons were then used to amplify a cDNA product that linked these exons. PCR reactions were fractionated on agarose gels, blotted to nylon membranes, and probed with a *cdo* cDNA probe. To analyze differential splicing of the fifth Ig repeat of *cdo* in Rat 6 cells, reverse transcription–PCR was performed on total Rat 6 cell RNA with the following primers: 5′-GGAAGCAACTGGAGAAGG-3′ (sense) and 5′-GTCTCGTTCATCGTTCTG-3′ (antisense). The products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Flow Cytometry

Cells were stained with propidium iodide buffer (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) in the presence of 10 μg of DNase-free RNase and analyzed by flow cytometry on a fluorescence-activated cell sorter (FACS®can; Becton and Dickinson, Co., Mountain View, CA). The data were analyzed by the CellFIT software program (Becton and Dickinson, Co., Mountain View, CA).

Antibodies and Western Blot Analyses

A portion of the intracellular region of rat *cdo* (amino acids 1057–1241) was ligated into pGEX-5X-1 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to produce a glutathione-*S*-transferase fusion protein. Fusion protein produced in *Escherichia coli* was purified by SDS-PAGE and electroelution. Rabbit polyclonal antibodies to CDO protein were generated with a proprietary immunization regimen referred to as PolyQuik™ (Zymed Laboratories Inc., South San Francisco, CA). Crude antisera were used for Western blot analyses.

Immunoblot analyses were performed essentially as described in Kang et al. (1996). Cells from monolayer or methylcellulose suspension cultures were harvested in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.2,150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/1 mM EGTA) containing 1 mM PMSF, 10 ng/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate. Total proteins were then separated by SDS-PAGE, transferred to nitrocellulose membranes (Amersham Corp., Arlington Heights, IL), and probed with anti-CDO antiserum. The blots were washed and reprobed with HRP-conjugated secondary antibody and specific protein bands visualized with the ECL chemiluminescent detection system (Amersham Corp.) as described previously (Kang, et al., 1996).

Surface Labeling and Tunicamycin Treatment

To surface biotinylate Rat 6–derived cultures, cells were detached in 0.04% EDTA in PBS, washed with serum-free DME, and incubated on ice with serum-free DME plus 50 μM sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) for 0.5 h. Cells were then washed again and lysed in RIPA buffer, and CDO was immunoprecipitated. Western blotting was then performed as described above, except that HRP-linked streptavidin was used for detection. To assess N-glycosylation of CDO, Rat 6 cells were treated with tunicamycin (5 μg/ml) for 48 h, lysed, and analyzed by Western blotting.

Ectopic Expression of cdo

A complete rat *cdo* open-reading frame, including 95 bp of the 5′ untranslated region and 633 bp of the 3′ untranslated region, was reconstructed from overlapping bacteriophage inserts and ligated into the retroviral expression vector, pBabePuro (Morganstern and Land, 1990). Production of recombinant retroviruses and infection of Rat 6–derived cell lines was performed as previously described (Krauss et al., 1992). Infected cultures were selected in medium that contained 5 μg puromycin per ml, and >1,000 independent, drug-resistant colonies were pooled and analyzed as described in Results.

Chromosomal Localization of Human cdo

A portion of the human *cdo* gene was PCR amplifed from human monochromosomal somatic cell hybrids. The National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel No. 2, version 3 (Coriell Institute for Medical Research, Camden, NJ), was used for chromosomes 1, 6–9, 11, 12, 14, 16, 17, 20, 22, X, and Y. The remaining chromosomes were represented by monochromosomal hybrids: GM11686 (chr. 2); GM11713 (chr. 3); GM11687 (chr. 4); GM11714 (chr. 5); GM11688 (chr. 10); GM11689 (chr. 13); GM11715 (chr. 15); GM12082 (chr. 18); GM10612 (chr. 19); and GM08854 (chr. 21) (Coriell Institute for Medical Research). PCR amplifications were performed with the following primers: 5′-CACAACAGGCTGGGAGCGA-3′ (sense), and 5′-GGGGAAGAAAACATTTAAG-3′ (antisense). The identity of the chromosome 11 monochromosomal hybrid was confirmed by PCR of the previously mapped locus catalase (11p13) (Theune et al., 1991). Human *cdo* was further localized with a chromosome 11 regional mapping panel (Coriell Institute for Medical Research).

Results

Isolation, Sequence Analysis, and Tissue Distribution of cdo

The R-1a and ER-1-2 mutant rat embryo fibroblast cell lines are resistant to induction of anchorage-independent growth by various oncogenes, and this property is dominant in somatic cell hybridizations (Krauss et al., 1992). Because this dominant resistance could have arisen by the stable expression (or overexpression) of a transformation suppressor gene, a cDNA library constructed from R-1a cells was screened by differential hybridization to isolate genes whose expression was enhanced in R-1a cells, relative to a transformation-sensitive control cell line, PKC3-F4. One cDNA clone isolated in this screen, later named *cdo*, hybridized to an ~8.5-kb mRNA species that reproducibly showed a mild (1.5–2-fold) up-regulation in both R-1a and ER-1-2 cells, relative to the control PKC3-F4 cells (Fig. 1). Interestingly, *cdo* mRNA levels were dramatically down-regulated in PKC3-F4 cells that had been infected with a v-H-*ras*–expressing retrovirus. This down-regulation was incomplete in similarly infected cultures of the *ras*-resistant R-1a and ER-1-2 cell lines (Fig. 1).

Sequence analysis of overlapping cDNAs revealed *cdo* to be a member of the Ig/FNIII repeat family. Fig. 2 *A* shows the predicted amino acid sequence of rat *cdo*; also shown is the amino acid sequence of a closely related human gene (see below). The rat *cdo* mRNA is predicted to encode a 1,242–amino acid protein, comprised of a 24–amino acid hydrophobic signal sequence followed by an extracellular region of five Ig V–like repeats (Vaughn and Bjorkman, 1996) and three FNIII-like repeats, a 25-residue, membrane-spanning region, and a putative intracellular region of 256 amino acids. A hydropathy plot (not shown) is consistent with a single membrane-spanning region, suggesting that *cdo* encodes a transmembrane protein with a structure similar to members of the N-CAM family. A schematic representation of the predicted CDO structure is shown in Fig. 2 *B*. While there are no other IgSF members with this numerical combination of Ig- and FNIII-like repeats in the current databases, individual Ig and FNIII repeats in *cdo* appeared to be most closely related to such

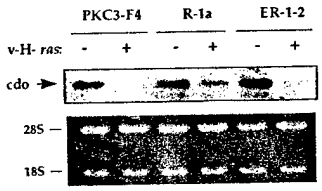

Figure 1. Northern blot analysis of *cdo* mRNA expression in the PKC3-F4, R-1a, and ER-1-2 cell lines, plus or minus infection with a v-H-*ras*–expressing retrovirus. + and −, expressing or not expressing v-H-*ras*, respectively. The ethidium bromide–stained gel (displaying the *28S* and *18S* ribosomal RNA bands), is shown as a loading control.

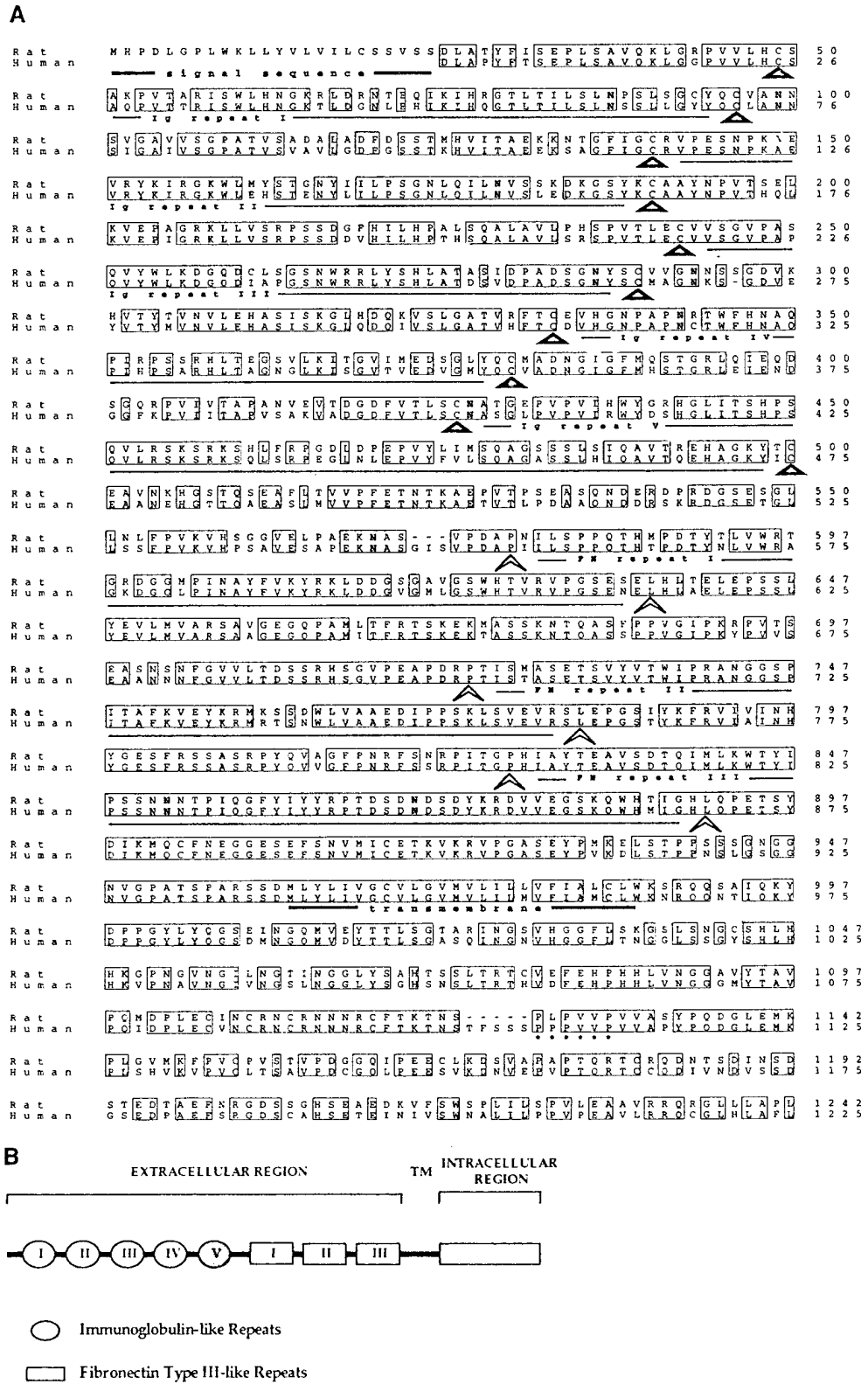

Figure 2. (*A*) Predicted amino acid sequence of rat and human CDO. Identical residues are boxed. The five Ig-like repeats are indicated by the triangles, which correspond to the two conserved cysteines present in each domain (Williams and Barclay, 1988). The three FNIII-like repeats are indicated by the arrowheads, which correspond to the conserved prolines and leucines at the end of each domain (Patthy, 1990). The putative signal sequence and transmembrane regions are underlined in bold. 10 asparagines in the extracellular region that represent potential sites of N-linked glycosylation are in bold print. A proline-rich stretch in the intracellular region is underscored with asterisks. These sequence data are available from EMBL/GenBank/DDBJ, under accession numbers AF004840 (rat), and AF004841 (human). (*B*) Schematic representation of CDO structure, which contains five Ig-like repeats, three FNIII-like repeats, a transmembrane region (*TM*), and a 256–amino acid intracellular region. Ig repeat 5 (*cross-hatched*) is alternatively spliced.

repeats found in rat NB-2 and NB-3 (Ogawa et al., 1996), members of the F11/contactin/F3 subfamily of CAMs (up to 50% amino acid similarity; data not shown). Interestingly, however, the putative intracellular region of *cdo* showed no significant resemblance to any gene currently in the standard databases. A proline-rich stretch in the intracellular region that might serve as a potential SH3 domain binding site is indicated in Fig. 2 *A*. 10 potential N-linked gycosylation sites present in the extracellular region are also indicated.

Sequencing of multiple, independent cDNAs revealed a potential for alternative splicing of the fifth Ig repeat,

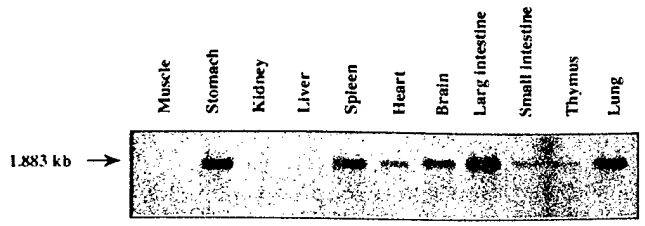

Figure 3. Expression of *cdo* in adult rat tissues. PCR-based "exon-connection" assays for detection of *cdo* transcripts were performed as described in the text.

leading to removal of amino acids 400–516. Reverse transcriptase–PCR analysis of total Rat 6 cell RNA with primers that flank this region produced a major product corresponding to mRNA that encodes the fifth Ig domain, and a minor product corresponding to mRNA that lacks this domain (data not shown). Consistent with the notion that this is a minor splice variant in Rat 6 cells, only one major form of *cdo* mRNA and protein was detected by Northern and Western blot analyses of these cells (see below).

Expression of *cdo* in adult rat tissues was analyzed initially by Northern blotting of poly(A)$^+$ RNA derived from brain, liver, kidney, heart, large and small intestines, spleen, thymus, lung, stomach, and skeletal muscle, but only a very faint signal could be detected. This result was reminiscent of that observed for the related gene, *DCC*. We chose, therefore, to use the "exon-connection" strategy originally used by Fearon et al. (1990) to analyze *DCC* expression. A product of the predicted size was found in all of the tissues examined except liver, kidney, and skeletal muscle (Fig. 3). Thus, *cdo* is expressed at low levels in most adult tissues.

Regulation of cdo mRNA by Oncogenes and Serum

The PKC3-F4, R-1a, and ER-1-2 cell lines all share the same original parental cell line, Rat 6. Expression of *cdo* mRNA in Rat 6 cells, as well as in Rat 6 cells transformed by different oncogenes, was therefore assessed. Transformation of Rat 6 cells by the H-*ras*, *neu*, v-*src*, v-*raf*, protein kinase C ϵ, or v-*fos* oncogenes led to a dramatic loss of expression of *cdo* (Fig. 4 *A*). Each of these oncogene-expressing cell lines was grossly morphologically transformed and formed large colonies in soft agar (Borner et al., 1992). In contrast, overexpression of protein kinase C β1, which led to enhanced mitogen-induced signaling but was a very weak transforming gene (Housey et al., 1988; Borner et al., 1995), had little effect on *cdo* expression. Furthermore, hybridization of a rat *cdo* cDNA probe to Northern blots of RNA from the murine embryo fibroblast line, NIH 3T3, revealed an mRNA species that was indistinguishable in size from that seen in Rat 6 cells, and this mRNA was nearly undetectable in NIH 3T3 cells transformed by a K-*ras* oncogene (Fig. 4 *B*). Thus, down-regulation of *cdo* mRNA expression correlated with establishment of the transformed phenotype by several different oncogenes in Rat 6 cells and by *ras* oncogenes in two different cell lines.

The possibility that *cdo* expression might be down-regulated by mitogen treatment was examined next. Fig. 4 *C* demonstrates that when confluent, serum-starved Rat 6

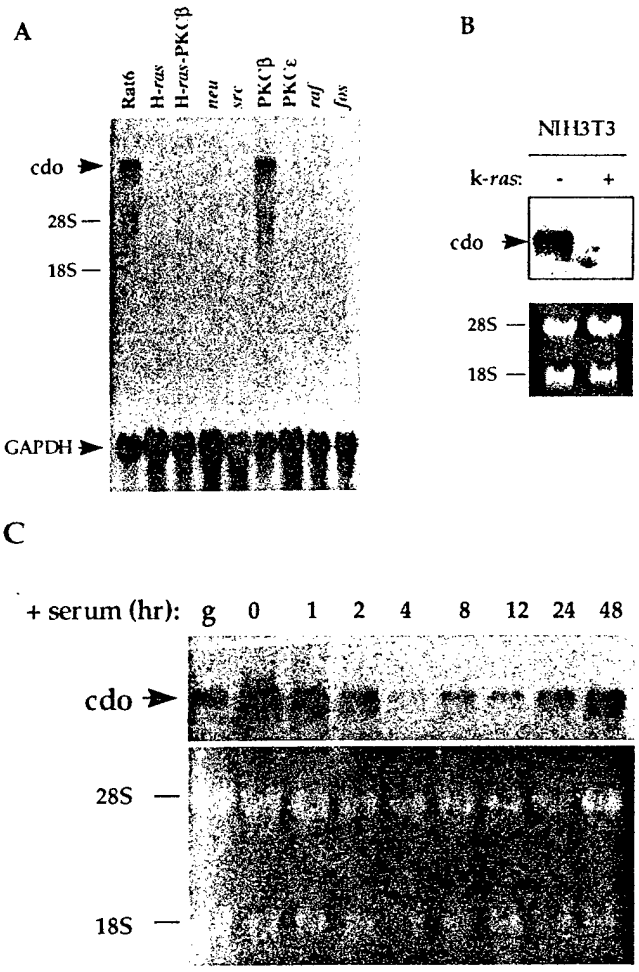

Figure 4. Effect of oncogenes and serum on *cdo* mRNA expression. (*A*) Northern blot analysis of *cdo* expression in Rat 6 cells and various oncogene-transformed Rat 6 cell derivatives. The lane designations refer to the oncogene expressed in the given Rat 6 cell derivative. The same blot was rehybridized with a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe as a loading control. (*B*) Northern blot analysis of *cdo* expression in NIH 3T3 cells, and NIH 3T3 cells transformed by the k-*ras* oncogene. + and −, expressing or not expressing k-*ras*, respectively. The ethidium bromide–stained gel, displaying the 28S and 18S ribosomal RNA bands, is shown as a loading control. (*C*) Northern blot analysis of the time course of *cdo* mRNA expression in serum-stimulated Rat 6 cells. Confluent cultures of wild-type Rat 6 cells were serum starved (*0 h*) and then fed with fresh, serum-containing medium. Lane designations represent the time, in hours, after serum stimulation; lane *g* represents cells growing logarithmically in serum-containing medium. The ethidium bromide–stained gel, displaying the 28S and 18S ribosomal RNA bands, is shown as a loading control.

cells were stimulated by refeeding with fresh, serum-containing medium, *cdo* expression was nearly extinguished within 4 h. Expression began to return within 8–12 h, and by 24–48 h, it had been restored to the level of the original cultures. Parallel cultures that were analyzed for cell cycle progression by flow cytometry indicated that >70% of the cells were in S phase 16 h after serum stimulation, and that the cultures were fully quiescent again 24–48 h after-stimulation (data not shown). Thus, steady-state levels of *cdo* mRNA were transiently down-regulated as G_o-arrested

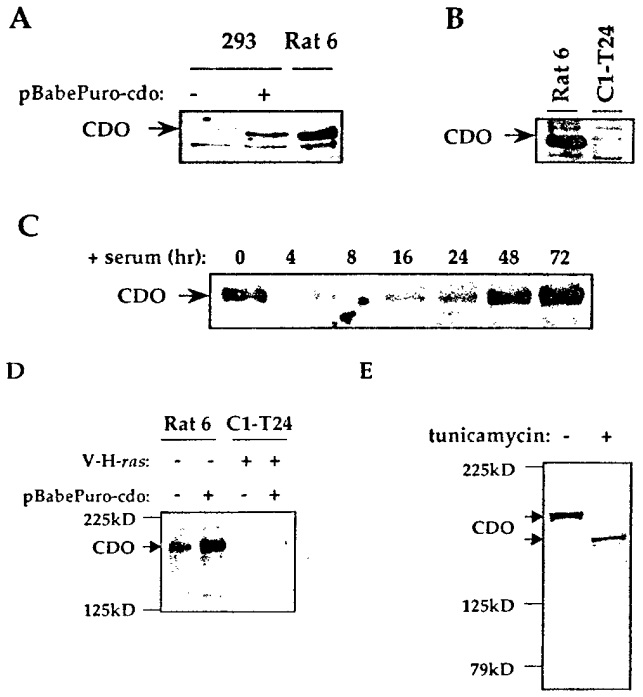

Figure 5. Western blot analyses of CDO protein expression with antisera against the CDO intracellular region. (*A*) Transient transfection of the pBabePuro/*cdo* vector into 293 cells. + and −, cells were transfected or mock transfected with the expression vector, respectively. (*B*) Ras-mediated down-regulation of CDO protein levels in Rat 6 cells. C1-T24 cells are a *ras*-transformed Rat 6 cell subclone. (*C*) Serum-mediated down-regulation of CDO protein levels in Rat 6 cells. Confluent cultures of wild-type Rat 6 cells were serum starved (*0 h*) and then fed with fresh, serum-containing medium. Lane designations represent the time, in hours, after serum stimulation; note that the times after stimulation at which CDO protein was analyzed are not identical to those used in analysis of *cdo* mRNA in Fig. 2. (*D*) Cell surface expression of CDO. Rat 6 and C1-T24 cell derivatives were surface biotinylated and detected on Western blots with HRP-linked streptavidin. + and −, cells did, or did not, stably express the pBabePuro/*cdo* vector. (*E*) N-linked glycosylation of CDO. + and −, Rat 6 cells treated or not treated with tunicamycin, respectively, and then analyzed by immunoblotting with CDO antisera.

cells reentered the cell cycle and were restored as the cultures regained quiescence. Fig. 4 *C* also shows that logarithmically growing cells had lower levels of *cdo* mRNA than did confluent, serum-starved cells. This appeared not to be due simply to cell proliferation, per se, or solely to the fact that these growing cultures were in the presence of serum, because growth arrest of such cultures by removal of serum did not significantly increase *cdo* expression (data not shown). These data indicate that cell density and/or cell–cell contact might also play a role in *cdo* mRNA regulation.

Analysis of CDO Protein in Rat 6 Cells

A complete *cdo* open reading frame was inserted into the retroviral expression vector, pBabePuro. This vector was transiently transfected into the human embryonic kidney cell line, 293, and Western blot analysis performed on total cell lysates with polyclonal rabbit antisera raised against the intracellular region of *cdo*. A protein of ~190 kD was detected in transfected cells, but not in mock-transfected controls (Fig. 5 *A*). An immunoreactive protein with similar mobility was detected in Rat 6 cell lysates (Fig. 5 *A*). A nonspecific, cross-reactive band of lower molecular weight is also visible in mock-transfected 293 cells and, frequently, in Rat 6 cells. Consistent with the RNA analyses described above, CDO protein levels were dramatically down-regulated in *ras*-transformed Rat 6 cells (C1-T24 cells; Fig. 5 *B*). Likewise, serum stimulation of quiescent Rat 6 cultures led to transient down-regulation of CDO protein levels (Fig. 5 *C*). It was not possible to perform similar studies with NIH 3T3 cells, because the antibodies to CDO appear to be specific for rat.

The amino acid sequence of *cdo* predicts a transmembrane protein. To confirm that CDO was expressed on the cell surface, various Rat 6 cell derivatives were surface biotinylated. CDO was then immunoprecipitated and detected on Western blots with HRP-coupled streptavidin. As shown in Fig. 5 *D*, a band of the appropriate molecular weight was observed in Rat 6 cells, but was not present in *ras*-transformed C1-T24 cells. Furthermore, the signal intensity of the band was increased in Rat 6 cells that overproduce CDO, and the band was restored in C1-T24 cells that ectopically produce this protein (see below for a description of these cell lines). It is concluded that CDO is expressed on the cell surface.

Members of the IgSF are typically modified in their extracellular region by N-linked glycosylation (Brummendorf and Rathjen, 1995). To test whether CDO is also N-glycosylated, Rat 6 cells were treated with tunicamycin, an inhibitor of this process. CDO from treated cells displayed a significant decrease in apparent molecular weight (Fig. 5 *E*), consistent with the notion that it is a glycoprotein.

Effect of Cell–Substratum Adhesion on cdo RNA and Protein Expression

The expression pattern of *cdo* in Rat 6 and NIH 3T3 cells (Fig. 4) suggests that *cdo* might encode a protein with growth inhibitory or transformation suppressor function. Because the primary defect in the R-1a and ER-1-2 mutant cell lines is an inability to proliferate in an anchorage-independent manner in response to *ras* (Krauss et al., 1992; Kang and Krauss, 1996), it was of interest to determine the effects of cell–substratum adhesion on the expression of *cdo*. Rat 6 cells suspended in methylcellulose-containing medium had significantly lower steady state levels of *cdo* mRNA than did parallel adherent cultures. A similar effect was observed in NIH 3T3 cells (Fig. 6). *cdo* mRNA levels were consistently lower in suspension cultures than in adherent cultures of low cell density (data not shown). It seems likely, however, that the decrease in steady state *cdo* mRNA levels seen in the methylcellulose cultures of Rat 6 cells may reflect a combination of two phenomena: (*a*) loss of cell anchorage; and (*b*) the low cell density typically used for these suspension cultures, since decreased cell density in adherent cultures also led to partially reduced levels of *cdo* mRNA (Fig. 4 *C*, and see above).

Although *cdo* mRNA levels were down-regulated in nonadherent Rat 6 cells, some level of expression was always

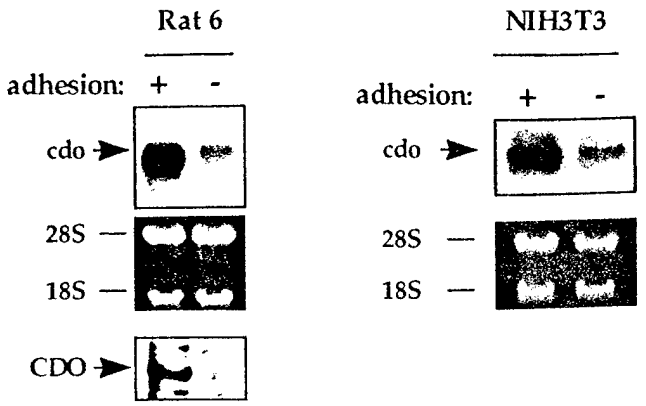

Figure 6. Regulation of *cdo* mRNA and protein by cell–substratum adhesion in Rat 6 and NIH 3T3 cells. + and −, cells were cultured on plastic dishes or suspended in methylcellulose-containing medium, respectively. CDO protein and *cdo* mRNA were analyzed by Western and Northern blotting techniques, respectively. For the Northern blots, the ethidium bromide–stained gel, displaying the 28S and 18S ribosomal RNA bands, is shown as a loading control.

detectable in these cultures. In contrast, CDO protein could not be detected in suspension cultures (Fig. 6). These data raised the possibility that CDO protein levels might be regulated by cell–substratum adhesion by a mechanism that is independent of *cdo* mRNA expression. Additional experiments that address this point are described below.

Ectopic Expression of cdo

To test the hypothesis that cell–substratum adhesion can regulate CDO protein levels independently of *cdo* mRNA expression, we sought to express a *cdo* cDNA from a promoter whose activity was not influenced by cell culture conditions. To achieve this, parental Rat 6 cells and a *ras*-transformed derivative, C1-T24, were infected with a recombinant retrovirus that drives expression of *cdo* from the viral long terminal repeat. The cells were infected with either the *cdo*-expressing virus or, as a control, a virus that lacked a cDNA insert. Infectants were then pooled and the polyclonal populations were analyzed.

Ectopic expression of *cdo* was assessed by Northern and Western blot analyses of infected cells cultured under both adherent and nonadherent conditions. As shown in Fig. 7 *A*, Rat 6 and C1-T24 cells that were infected with the *cdo* virus (Rat 6/*cdo* and C1-T24/*cdo* cells, respectively) expressed abundant, vector-derived mRNA whether they were attached to plates or suspended in methylcellulose. When adherent cultures were tested, Rat 6/*cdo* cells produced significantly more immunoreactive CDO protein than control vector-infected cells (Fig. 7 *A*). C1-T24/*cdo* cells also produced large quantities of CDO protein when grown on tissue culture plates (Fig. 7 *A*). Control vector-infected C1-T24 cells expressed no detectable CDO, similar to the parental C1-T24 cell line shown in Fig. 5 *B* (see also Fig. 7 *B*). Interestingly, no CDO protein was produced when any of these cell lines was cultured under nonadherent conditions, even though they stably expressed exogenous, vector-derived *cdo* mRNA (Fig. 7 *A*). We have previously

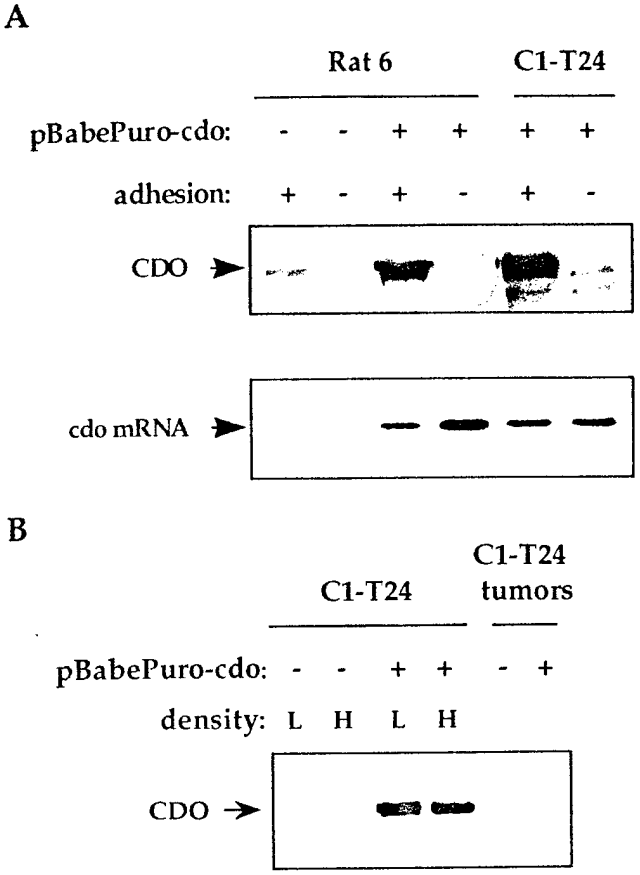

Figure 7. Regulation of ectopic CDO protein production by cell–substratum adhesion. (*A*) Rat 6 and C1-T24 cells were infected with recombinant pBabePuro/*cdo* virus (+) or pBabePuro virus lacking a cDNA insert (−), and were cultured on plastic dishes (+ *adhesion*) or suspended in methylcellulose-containing medium (− *adhesion*). CDO protein expression and mRNA expression were analyzed by Western and Northern blotting techniques, respectively. Prolonged exposure of the Northern blot revealed the endogenous *cdo* mRNA species in the first two lanes, similar to the data presented in Fig. 6. (*B*) C1-T24 cells were infected with recombinant pBabePuro/*cdo* virus (+) or pBabePuro virus lacking a cDNA insert (−), and were then cultured on plastic dishes at low (*L*) or high (*H*) cell density. Also shown are extracts from tumors derived from C1-T24/*puro* (−) and C1-T24/*cdo* (+) cells. Samples were analyzed for CDO protein expression by Western blotting techniques. Western blots were also stained with Ponceau S to confirm that protein samples were not degraded.

demonstrated that production of several cell cycle proteins is not altered by culturing these cells in methylcellulose-containing medium (Kang and Krauss, 1996). Furthermore, immunoblotting of these same cell extracts with antibodies to a different cell surface protein, the insulin-like growth factor I receptor, did not reveal significant differences between adherent and nonadherent cultures in the expression of this protein (data not shown). Loss of CDO production in nonadherent cultures therefore was not due to a nonspecific effect, nor was it due to undetected damage to the cells. These data are consistent with the data described in Fig. 5, and indicate a requirement, at least in Rat 6 cells, for cell–substratum adhesion for stable production of CDO protein.

The failure of Rat 6 cells to produce either endogenous or ectopically expressed CDO protein in the absence of cell–substratum adhesion might have arisen, at least in part, as a result of the low cell density in the methylcellulose cultures used for the nonadherent conditions, rather than as an absolute need for cell anchorage. To distinguish between these possibilities, we cultured C1-T24/*cdo* cells at low and high cell densities on tissue culture plates and measured CDO levels by immunoblotting. C1-T24/*cdo* cells are most appropriate for addressing this question because they express only the vector-derived *cdo* mRNA which, unlike the endogenous *cdo* gene, is not regulated by cell density or cell anchorage. As shown in Fig. 7 *B*, adherent cultures of C1-T24/*cdo* cells harvested at low and high cell densities each produced abundant CDO protein, indicating that loss of cell substratum adhesion was most likely to be responsible for the effects observed in Fig. 7 *A*.

As would be expected of cells that failed to produce the exogenously derived protein, there was no difference between C1-T24/*cdo* cells and control vector-infected C1-T24 cells in their frequency of colony formation in soft agar (~20% cloning efficiency). The tumorigenicity of C1-T24/*cdo* and control vector-infected C1-T24 cells was also tested. When 10^6 cells of each cell line were injected into the flanks of nude mice, both produced tumors of 1-cm diam in 10 d. The tumors were then excised, and tested for expression of CDO protein. Similar to what was observed with the nonadherent cultures, tumors derived from C1-T24/*cdo* cells produced barely detectable levels of CDO (Fig. 7 *B*), further indicating a need for engagement of the cells with an appropriate substratum for stable production of this protein.

Finally, ectopic expression of CDO protein in adherent cultures of Rat 6/*cdo* and C1-T24/*cdo* cells did not alter the gross morphology or growth properties of either cell type when compared with their respective vector control or parental cell lines (data not shown). Notably, no increase in cell–cell aggregation was observed, as might be expected for ectopic expression of a cell surface molecule that mediated homophilic cell adhesion.

It should be noted that the *ras*-resistant mutant cells also did not produce CDO protein when cultured in suspension (data not shown). Thus, the failure of the mutant cell lines to down-regulate *cdo* mRNA levels to the same extent observed in transformation-sensitive control cells presumably does not account for their resistance to anchorage-independent growth. These data do not, however, exclude a role for CDO as a transformation suppressor in other systems where stable expression of the protein may occur.

Sequence and Chromosomal Localization of a Human Gene Closely Related to cdo

A Southern "zoo" blot containing DNA from various species revealed that mouse and human DNA displayed strong cross-species hybridization to a rat *cdo* cDNA probe, while chicken, toad (*Xenopus laevis*), and zebrafish DNA displayed a clearly detectable, but weaker signal (data not shown). Screening of human fetal brain and fetal lung libraries yielded clones that corresponded to a single closely related gene. These human cDNAs hybridized to an mRNA species present in several human neuroblastoma cell lines that was indistinguishable in size from *cdo* mRNA in Rat 6 cells (data not shown).

The predicted amino acid sequence of this human gene is shown in comparison to that of rat *cdo* in Fig. 2 *A*. The human and rat genes have identical domain structures in their extracellular regions, and their intracellular regions, which do not resemble other known proteins, are also highly related. Across their full lengths (except for the signal sequence which was missing from the human cDNA clones obtained), the two proteins are ~81% identical and ~96% similar. The relative positions of amino acids that define the Ig- and FNIII-like domain consensus sequences are conserved between the two predicted proteins, as are all 10 potential N-linked glycosylation sites. Two potential microexons are apparent in the human sequence: one is a three–amino acid insertion between Ig-like repeat 5 and FNIII-like repeat 1; the second is a five–amino acid insertion that is immediately adjacent to the proline-rich sequence found in the cytoplasmic tail. Although we cannot rule out the existence of human genes that are even more closely related to rat *cdo*, in this article, this gene will be referred to as human *cdo*.

To identify a chromosomal location for human *cdo*, a panel of human/rodent monochromosomal hybrids was screened by PCR with primers that amplify human, but not rodent, *cdo* DNA. These studies mapped human *cdo* to chromosome 11 (data not shown). PCR analysis of a chromosome 11 regional mapping panel showed that *cdo* mapped to the interval 11q23–24 as defined by the break-

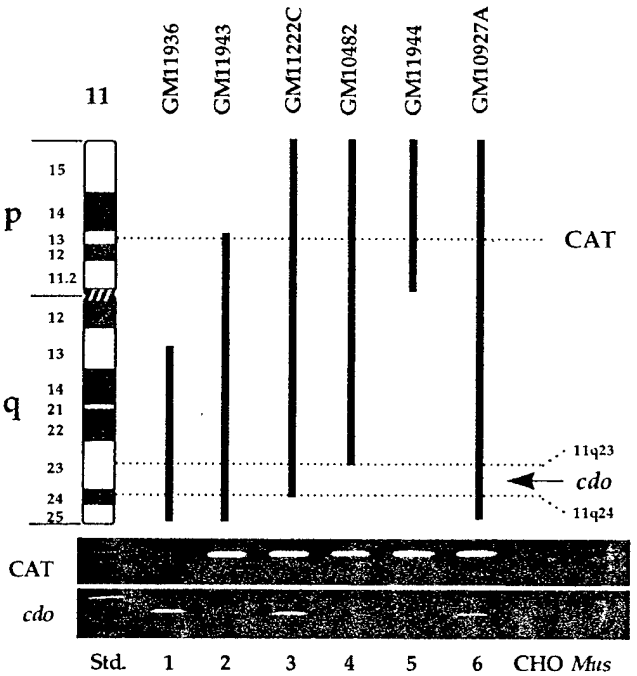

Figure 8. Regional localization of human *cdo* to chromosome 11q23–24. PCR amplification of human *cdo* and, as a control, the catalase locus (*CAT*; *11p13*) in a regional mapping panel containing the displayed portions of human chromosome 11 in a rodent cell background. Ethidium bromide–stained gels are shown. *CHO*, genomic DNA from CHO cells; *Mus*, genomic DNA from mouse.

points of the hybrids GM10482 (11q23) and GM11222C (11q24). Hybrid GM11222C was positive for *cdo*, whereas GM10482 was negative (Fig. 8). This region has substantial overlap with similarly sized regions that display LOH in a high percentage of human breast, ovarian, and lung cancers (Negrini et al., 1995; Rasio et al., 1995; Gabra et al., 1996). PCR for the catalase locus (11p13) was used as a control to confirm the identity of the hybrids (Fig. 8).

Discussion

Proteins of the Ig/FNIII family play important roles in a variety of developmental and physiological processes. Additionally, expression of Ig/FNIII proteins can be highly dynamic and cell type specific, although little is known of the signaling pathways that regulate such expression (Edelman and Crossin, 1991; Brummendorf and Rathjen, 1995; Cunningham, 1995). We report here the molecular cloning and characterization of rat CDO, a novel cell surface glycoprotein of the Ig/FNIII family. A *cdo* cDNA was isolated by screening a genetically dominant, oncogene-resistant mutant cell line for genes whose expression was enhanced relative to a transformation-sensitive control cell line. *cdo* mRNA and protein were dramatically down-regulated in rat and mouse embryo fibroblast cell lines that were transformed by various oncogenes. Additionally, *cdo* mRNA and protein levels were strongly, but transiently, reduced when confluent, quiescent cells were stimulated to reenter the cell cycle. These observations raise the possibility that CDO may serve as a negative regulator of cell proliferation, transformation, and/or tumorigenesis. Consistent with this possibility, a human gene that is very closely related to rat *cdo* mapped to chromosome 11q23–24, a region that exhibits LOH in a high percentage of human breast, ovarian, and lung cancers (Negrini et al., 1995; Rasio et al., 1995; Gabra et al., 1996). *cdo* therefore joins a growing list of genes that are not only down-regulated at the mRNA level in transformed cell lines or tumors, but may also have transformation suppressor function. Such genes include those that encode components of the extracellular matrix (fibronectin, collagen); cell surface proteins (E- and H-cadherin, connexins 26 and 43, certain integrins, and maspin); and proteins that organize the actin cytoskeleton (vinculin, α-actinin, tropomyosin 1) (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990; Frixen et al., 1991; Vleminckx et al., 1991; Fernandez et al., 1992; Gluck et al., 1993; Prasad et al., 1993; Akamatsu et al., 1996; Hirschi et al., 1996; Lee, 1996; Sheng et al., 1996; Travers et al., 1996).

In addition to regulation of *cdo* mRNA levels by oncogenes and serum, CDO protein levels were powerfully controlled by cell–substratum adhesion, via a mechanism that was independent of *cdo* mRNA expression. Rat 6 cells and the Ras-transformed derivative cell line C1-T24 each displayed a cell anchorage requirement for stable production of CDO, even when this protein was derived from an ectopically expressed mRNA, whose levels were not altered by the culture conditions. Preliminary experiments indicate that when suspended C1-T24/*cdo* cells were allowed to attach and spread on fibronectin-coated dishes, CDO was restored to maximal levels 2 h after replating. Furthermore, attachment-mediated production of CDO was partially blocked by cytochalasin D (Kang, J.-S., and R.S. Krauss, unpublished observations). These data suggest a possible role for integrin-mediated signaling and cytoskeletal organization in this process. They also raise the possibility that stable production of CDO may require an appropriate extracellular matrix microenvironment. Such a requirement would have important implications for cell type specificity in the production of CDO and other proteins that might be regulated in a similar fashion. It will be very interesting to determine whether anchorage or specific extracellular matrix proteins are required for stable production of other Ig/FNIII family members. It is not known whether the loss of CDO protein in suspension cultures occurred at the level of translation or stability. Zhu et al. (1996) have reported that translation of cyclin D1 mRNA is dependent on cell–substratum adhesion, but unlike the observations described here for CDO, this effect of cell anchorage was lost when ectopic cyclin D1 transcripts lacked untranslated regions.

Most CAMs of the IgSF can mediate homophilic binding, although DCC may be an exception (Fearon, 1996). However, Rat 6 and C1-T24 cells that ectopically expressed CDO did not exhibit enhanced cell–cell aggregation. Furthermore, preliminary studies indicate that a soluble fusion protein that contains the entire CDO extracellular region is unable to bind to Rat 6 or Rat 6/*cdo* cells, each of which has abundant CDO on their cell surface (Kang, J.-S., and R.S. Krauss, unpublished observations). These observations indicate that Rat 6 cells do not express a ligand for CDO on their cell surface, and also that CDO is probably not á homophilic CAM. Therefore, it will also be interesting to determine the effects of cell anchorage on CDO production in a cell system in which a known ligand for CDO is present. The presence of a ligand could conceivably stabilize or stimulate production of CDO, even in the absence of optimal cell anchorage. The levels of CDO at the cell surface may therefore be governed by complex interactions between (*a*) signals that can regulate *cdo* gene expression, such as mitogenic stimuli; and (*b*) signals that can regulate CDO protein production, such as appropriate cell–substratum interactions and, possibly, the presence of ligands.

Members of the Ig/FNIII repeat family can be organized into subfamilies by the number of Ig- and FNIII-like repeats they contain in their extracellular regions (Brummendorf and Rathjen, 1995; Cunningham, 1995). We are not aware of any previously reported members of this family that have the five Ig plus three FNIII combination of repeats. CDO's unique intracellular region also distinguishes it from other Ig/FNIII proteins. *cdo* is therefore a founding member of a new subfamily within the Ig/FNIII repeat family. Given the structural resemblance of CDO to N-CAM, DCC, and other Ig/FNIII family members, it is tempting to speculate that CDO might also play a role in cell migratory events during development of the nervous system and/or other organ systems. Consistent with this notion, *cdo* mRNA is expressed at high levels in several human neuroblastoma cell lines (Gao, M., and R.S. Krauss, unpublished observations); in addition, preliminary in situ hybridization studies indicate that *cdo* mRNA is expressed at high levels in particular regions of the developing rat spinal cord and, interestingly, limb bud (Brose, K., J.-S.

Kang, R.S. Krauss, and M. Tessier-Lavigne, unpublished studies). Because under some conditions Rat 6 cells express *cdo* mRNA but not its protein product, it will be important to identify cell surface production of CDO protein by particular cell types during development to understand its function. Understanding CDO's possible functions in development and oncogenesis will also require analysis of the protein's long cytoplasmic tail, which bears no significant resemblance to other known proteins. The dramatic regulation of *cdo* mRNA and protein production by mitogenic and anchorage-related stimuli, combined with CDO's unique intracellular region, indicate that further studies of this protein should yield interesting new insights into cell proliferation and/or motility, and how such processes may go awry during tumorigenesis. Such studies are in progress.

We thank C. Goodman, T. Kidd, and M. Tessier-Lavigne for communicating results before publication; E. Spanopoulou for cell lines; and L. Ossowski, J. Hirsch, and D. Colman for comments on the manuscript.

This work was supported by grants from the National Intstitutes of Health (CA59474) and the Elsa U. Pardee Foundation, as well as a scholarship from the Alexandrine and Alexander L. Sinsheimer Fund to R.S. Krauss. J.L. Feinleilb is supported by a U.S. Army Medical Research and Materiel Command Breast Cancer predoctoral training grant (DAMD17-94-J-4111). R.S. Krauss is a Career Scientist of the Irma T. Hirschl Trust.

Received for publication 29 January 1997 and in revised form 26 April 1997.

References

Akamatsu, H., K. Ichihara-Tanaka, K. Ozono, W. Kamiike, H. Matsuda, and K. Sekiguchi. 1996. Suppression of the transformed phenotypes of human fibrosarcoma cells by overexpression of recombinant fibronectin. *Cancer Res.* 56: 451–456.

Aoki, J., M. Umeda, K. Takio, K. Titani, H. Utsemi, M. Sasaki, and K. Inoue. 1991. Neural cell adhesion molecule mediates contact-dependent inhibition of growth of near-diploid mouse fibroblast cell line m5S/1M. *J. Cell Biol.* 115:1751–1761.

Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408–1412.

Berx, G., A.-M. Cleton-Jansen, K. Strumane, W.J.F. de Leeuw, F. Nollet, F. van Roy, and C. Cornelisse. 1996. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene.* 13:1919–1925.

Borner, C., S.N. Guadagno, W.W.-L. Hsiao, D. Fabbro, M. Barr, and I.B. Weinstein. 1992. Expression of four protein kinase C isoforms in rat fibroblasts. Differential alterations in *ras*-, *src*-, and *fos*- transformed cells. *J. Biol. Chem.* 267:12900–12910.

Borner, C., M. Ueffing, S. Jaken, P.J. Parker, and I.B. Weinstein. 1995. Two closely related isoforms of protein kinase C produce reciprocal effects on the growth of rat fibroblasts. *J. Biol. Chem.* 270:78–86.

Brummendorf, T., and F.G. Rathjen. 1995. Cell adhesion molecules 1: immunoglobulin superfamily. *Protein Profile.* 2:963–1108.

Chan, S.S.-Y., H. Zheng, M.-W. Su, R. Wilk, M.T. Killeen, E.M. Hedgecock, and J.G. Culotti. 1996. UNC-40, a *C. elegans* homologue of DCC (deleted in colorectal cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell.* 87:187–195.

Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Coman, D.R. 1944. Decreased mutaul adhesiveness, a property of cells from squamous cell carcinomas. *Cancer Res.* 4:625–629.

Cunningham, B.A. 1995. Cell adhesion molecules as morphoregulators. *Curr. Opin. Cell. Biol.* 7:628–633.

Edelman, G.M., and K.L. Crossin. 1991. Cell adhesion molecules: implications for a molecular histology. *Annu. Rev. Biochem.* 60:155–190.

Fearon, E.R. 1996. *DCC*: is there a connection between tumorigenesis and cell guidance molecules? *Biochim. Biophys. Acta.* 1288:M17–M23.

Fearon, E.R., and W.E. Pierceall. 1995. The *deleted in colorectal cancer* (*DCC*) gene: a candidate tumor suppressor gene encoding a cell surface protein with similarity to the neural cell adhesion molecules. *Cancer Surv.* 24:3–17.

Fearon, E.R., K.R. Cho, J.M. Nigro, S.E. Kern, J.W. Simons, J.M. Ruppert, S.R. Hamilton, A.C. Preisinger, G. Thomas, K. Kinzler, et al. 1990. Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science (Wash. DC).* 247:49–56.

Feinleib, J.L., and R.S. Krauss. 1996. Dissociation of *ras* oncogene-induced gene expression and anchorage-independent growth in a series of somatic cell mutants. *Mol. Carcinog.* 16:139–148.

Fernandez, J.L.R., B. Geiger, D. Salomon, H. Sababay, M. Zoller, and A. Ben-Ze'ev. 1992. Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA. *J. Cell. Biol.* 119:427–438.

Frixen, U.H., J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Lochner, and W. Birchmeir. 1991. E-cadherin–mediated cell–cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113:173–185.

Gabra, H., J.E.V. Watson, K.J. Taylor, J. Mackay, R.C.F. Leonard, C.M. Steel, D.J. Porteous, and J.F. Smyth. 1996. Definition and refinement of a region of loss of heterozygosity at 11q23.3-q24.3 in epithelial ovarian cancer associated with poor prognosis. *Cancer Res.* 56:950–954.

Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.

Gluck, U., D.J. Kwiatkowski, and A. Ben-Ze'ev. 1993. Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with α-actinin cDNA. *Proc. Natl. Acad. Sci. USA.* 90:383–387.

Hedrick, L., K.R. Cho, and B. Vogelstein. 1993. Cell adhesion molecules as tumour suppressors. *Trends Cell Biol.* 3:36–39.

Hedrick, L., K.R. Cho, E.R. Fearon, T.-C. Wu, K.W. Kinzler, and B. Vogelstein. 1994. The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes & Dev.* 8:1174–1183.

Hirschi, K.K., C.-E. Xu, T. Tsukamoto, and R. Sager. 1996. Gap junction genes *Cx26* and *Cx43* individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell Growth Differ.* 7:861–870.

Housey, G.M., M.D. Johnson, W.-L.W. Hsiao, C.A. O'Brian, J.P. Murphy, P. Kirschmeir, and I.B. Weinstein. 1988. Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. *Cell.* 52:343–354.

Hsieh, J.-T., W. Luo, W. Song, Y. Wang, D.I. Kleinerman, N.T. Van, and S.-H. Lin. 1995. Tumor suppressive role of an androgen-regulated epithelial cell adhesion molecule (C-CAM) in prostate carcinoma cell revealed sense and anti-sense approaches. *Cancer Res.* 55:190–197.

Hynes, R.O., and A.D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell.* 68:303–322.

Johnson, M.D., G.M. Housey, P.T. Kirschmeir, and I.B. Weinstein. 1987. Molecular cloning of gene sequences regulated by tumor promoters and mitogens through protein kinase C. *Mol. Cell. Biol.* 7:2821–2829.

Kang, J.-S., and R.S. Krauss. 1996. Ras induces anchorage-independent growth by subverting multiple adhesion-regulated cell cycle events. *Mol. Cell. Biol.* 16:3370–3380.

Keino-Masu, K., M. Masu, L. Hinck, E.D. Leonardo, S.S.-Y. Chan, J.G. Culotti, and M. Tessier-Lavigne. 1996. *Deleted in colorectal cancer* (*DCC*) encodes a netrin receptor. *Cell.* 87:175–185.

Kleinerman, D.I., P. Troncoso, S.-H. Lin, L.L. Pisters, E.R. Sherwood, T. Brooks, A.C. von Eisenbach, and J.-T. Hsieh. 1995. Consistent expression of an epithelial cell adhesion molecule (C-CAM) during human prostate development and loss of expression in prostate cancer: implication as a tumor suppressor gene. *Cancer Res.* 55:1215–1220.

Kolodziej, P.A., L.C. Timpe, K.J. Mitchell, S.R. Fried, C.S. Goodman, L.Y. Jan, and Y.N. Jan. 1996. *frazzled* encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell.* 87:197–204.

Krauss, R.S., S.N. Guadagno, and I.B. Weinstein. 1992. Novel revertants of H-ras oncogene-transformed R6-PKC3 cells. *Mol. Cell. Biol.* 12:3117–3129.

Kunath, T., C. Ordonez-Garcia, C. Turbide, and N. Beauchemin. 1995. Inhibition of colonic tumor cell growth by biliary glycoprotein. *Oncogene.* 11: 2375–2382.

Lee, S.W. 1996. H-cadherin, a novel cadherin with growth inhibitory functions and diminished expression in human breast cancer. *Nat. Med.* 2:776–782.

Morganstern, J.P., and H. Land. 1990. Advanced mammalian gene transfer: high titer retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587–3596.

Negrini, M., D. Rasio, G.M. Hampton, S. Sabbioni, S. Rattan, S.L. Carter, A.L. Rosenberg, G.F. Schwartz, Y. Shiloh, W.K. Cavenee, et al. 1995. Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. *Cancer Res.* 55:3003–3007.

Neumaier, M., S. Paululat, A. Chan, P. Matthaes, and C. Wagener. 1993. Biliary glycoprotein, a potential human cell adhesion molecule, is down-regulated in colorectal carcinomas. *Proc. Natl. Acad. Sci. USA.* 90:10744–10748.

Ogawa, J., H. Kaneko, T. Masuda, S. Nagata, H. Hosoya, and K. Watanabe. 1996. Novel neural adhesion molecules in the contactin/F3 subgroup of the immunoglobulin superfamily: isolation and characterization of the cDNAs from rat brain. *Neurosci. Lett.* 218:173–176.

Patthy, L. 1990. Homology of a domain of the growth hormone prolactin receptor with type III modules of fibronectin. *Cell.* 61:13–14.

Plantefaber, L.C., and R.O. Hynes. 1989. Changes in integrin receptors on oncogenically transformed cells. *Cell.* 56:281–290.

Prasad, G.L., R.A. Fuldner, and H.L. Cooper. 1993. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the *ras* oncogene. *Proc. Natl. Acad. Sci. USA.* 90:7039–7043.

Rasio, D., M. Negrini, G. Manetti, T.A. Dragani, and C.M. Croce. 1995. Loss of heterozygosity at chromosome 11q in lung adenocarcinoma: identification of three independent regions. *Cancer Res.* 55:3988–3991.

Sheng, S., J. Carey, E.A. Seftor, L. Dias, M.J.C. Hendrix, and R. Sager. 1996. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc. Natl. Acad. Sci. USA.* 93:11669–11674.

Shibata, D., M.A. Reale, P. Lavin, M. Silverman, E.F. Fearon, G. Steele, J.M. Jessup, M. Loda, and I.C. Summerhayes. 1996. The DCC protein and prognosis in colorectal cancer. *N. Engl. J. Med.* 335:1727–1732.

Sporns, O., G.M. Edelman, and K.L. Crossin. 1995. The neural cell adhesion molecule (N-CAM) inhibits proliferation in primary cultures of rat astrocytes. *Proc. Natl. Acad. Sci. USA.* 92:542–546.

Tessier-Lavigne, M., and C.S. Goodman. 1996. The molecular biology of axon guidance. *Science (Wash. DC).* 274:1123–1133.

Theune, S., J. Fung, S. Todd, A.Y. Sakaguchi, S.L. Naylor. 1991. PCR primers for human chromosomes: reagents for the rapid analysis of somatic cell hybrids. *Genomics.* 9:511–516.

Travers, H., N.S. French, and J.D. Norton. 1996. Suppression of tumorigenicity in ras-transformed fibroblasts by α2(1) collagen. *Cell Growth Differ.* 7:1353–1360.

Vaughn, D.E., and P.J. Bjorkman. 1996. The (Greek) key to structures of neural adhesion molecules. *Neuron.* 16:261–273.

Vleminckx, K., J. Vakaert, L., M. Mareel, W. Fiers, and F. Van Roy. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell.* 66:107–119.

Williams, A.F., and A.N. Barclay. 1988. The immunoglobulin superfamily: domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381–405.

Zhu, X., M. Ohtsubo, R.M. Bohmer, J.M. Roberts, and R.K. Assoian. 1996. Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J. Cell. Biol.* 133:391–403.